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PLAQUE ASSAY, CONCENTRATION AND CHEMICAL STUDIES  
OF A CANINE ADENOVIRUS, TORONTO A26/61

A THESIS

Submitted to the Faculty of Graduate Studies  
in Partial Fulfilment of the Requirements  
for the Degree of  
MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY  
FACULTY OF SCIENCE

by

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Edmonton, Alberta

January, 1966



UNIVERSITY OF ALBERTA  
FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Plaque Assay, Concentration and Chemical Studies of a Canine Adenovirus, Toronto A26/61" submitted by Roderick Allan Gaunt, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.



## ABSTRACT

A canine adenovirus, Toronto A26/61, responsible for the disease laryngotracheitis in dogs, was found to propagate equally well in both dog kidney cell line (DKL) monolayer cell cultures and primary dog kidney cell (PDK) monolayer cultures. Suspended cultures of DKL were unsatisfactory for the propagation of Toronto A26/61 virus. Variation of the concentration of Toronto A26/61 virus inoculum from 1 tissue culture infectious dose 50% end point per ml. ( $TCID_{50}$ /ml.) to 150 tissue culture infectious doses 50% end point per ml. had little effect on the ultimate virus yield in monolayer cultures of DKL cells.

The viruses Toronto A26/61, Infectious Canine Hepatitis (ICH), and Hepatitis Contagiosum Canis (HCC) produced hemagglutinins against the erythrocytes of human blood types, A, B, AB, O, and any one viral suspension gave an equal, reciprocal hemagglutinin titre with the four types of human erythrocytes. The hemagglutinin produced against human erythrocytes type "O" by Toronto A26/61 virus was inhibited by specific antiserum derived from rabbits previously infected with Toronto A26/61 virus. Toronto A26/61 virus produced intranuclear inclusions in DKL and PDK monolayer cell cultures which became evident 36 hours post-infection. The inclusions began as eosinophilic centers surrounded by basophilic borders and terminated as complete basophilic masses within the nucleus of each infected cell.





Toronto A26/61 virus was assayed by the TCID<sub>50</sub> method and/or by the plaque forming unit (PFU) method. Doubling the amino acid content in Eagle's medium, together with the addition of DEAE-dextran or protamine sulfate, was found to both maintain viability of the cells and to facilitate the production of larger plaques. Overlaid monolayers maintained in a carbon dioxide incubator (5% CO<sub>2</sub>, 95% filtered air) were found to remain viable for 18 days while those maintained in stoppered bottles remained viable for 8 - 12 days.

Under the conditions tested plaque production by Toronto A26/61 virus was better on DKL cells than on PDK cells. Of the five procedures examined for the concentration of Toronto A26/61 virus, namely:

- 1) differential centrifugation;
- 2) column chromatography on DEAE-cellulose;
- 3) Sephadex G200 filtration;
- 4) tris-genetron extraction of infected cells; and
- 5) buoyant-density centrifugation

differential centrifugation combined with buoyant-density centrifugation gave the best yield of virus as revealed by titration and ultraviolet spectrophotometric scan (200 mu. to 340 mu.). The buoyant-density of Toronto A26/61 virus was found to lie between 1.330 and 1.334 gms./cu. cm. when centrifuged to equilibrium in cesium chloride (CsCl). The



canine adenovirus (1CH) was found to have a similar buoyant-density of 1.333 gms./cu. cm. The buoyant-density of Toronto A26/61 virus deoxyribonucleic acid (DNA) centrifuged to equilibrium was found to be 1.718 gms./cu. cm. This buoyant-density corresponds to a mole fraction % guanine plus cytosine (G + C) of 59%, which is in contrast to 56% determined by direct chemical analysis. The buoyant-density of DNA extracted from DKL cells was found to be 1.701 gms./cu. cm. which corresponds to a mole fraction % (G + C) of 42%. This percentage was substantiated by a chemical analysis of the DKL-DNA which also revealed a mole fraction % (G + C) of 42%.

The chemical analysis of Toronto A26/61 virus DNA revealed a purine and pyrimidine base ratio of 27: 29: 22: 22 for guanine, cytosine, adenine, and thymine respectively. The chemical analysis of DKL-DNA revealed a purine and pyrimidine base ratio of 20: 22: 29: 29 for guanine, cytosine, adenine, and thymine respectively.



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At the University of Toronto during the fall of 1961 an epidemic of laryngotracheitis among experimental dogs was investigated by Ditchfield et al (1962). The illness was characterized by a dry cough (which was worse at night), a low-grade fever (102°F - 103°F), partial loss of appetite, and inflammation and edema of the pharynx, larynx and trachea. Pneumonia and/or conjunctivitis were complications to the condition. Spontaneous resolution usually occurred in seven to twelve days. Throat secretions from these dogs caused cytopathic effects on dog kidney line cells as well as on primary dog kidney cell monolayer cultures in vitro.

The cytopathic effect (CPE) was characterized by rounding of the cells with subsequent detachment from the surface of the vessel in which they were propagated.

Ditchfield et al (1962), by means of the complement-fixation test, neutralization test, and hemagglutination-inhibition test compared an agent which they isolated and designated as Toronto A26/61 to the only other described adenovirus of canine origin, infectious canine hepatitis, and a human adenovirus type 3 responsible for pharyngoconjunctival fever. The hemagglutination-inhibition test differentiated between the agents of infectious canine hepatitis and laryngotracheitis. Adenovirus type 3 possessed complement-fixing antibodies against all three viruses



(Toronto A26/61, ICH, type 3). Cross reactivity with respect to neutralizing antibody was observed between Toronto A26/61 and ICH viruses. Because of the foregoing criteria the etiological agent of laryngotracheitis was tentatively identified as a canine adenovirus.

Rowe et al (1958a) suggested that the adenovirus group should be divided into strains of human, chimpanzee, and monkey origin with a separate series of type numbers for each division. For example, the chimpanzee type would be named adenovirus type C-1 and monkey types M-1 to M-4. The monkey types have since been extended to 18.

Accepting this nomenclature, Ditchfield et al (1962) named infectious canine hepatitis virus as adenovirus type D-1 and Toronto A26/61 virus as either adenovirus type D-1A or, more justifiably, D-2.

Pereira (1959) has listed the main characteristics which he considers important to the classification of the adenovirus group: (1) ether resistance; (2) the ability to produce CPE in species specific tissue culture cells; and (3) the possession of a soluble group-specific complement-fixing antigen and lack of pathogenicity for ordinary laboratory animals. In addition, Parker et al (1961) suggested as another criterion that the basic infective particle must be a regular icosahedron between 80 to 120 mu. in diameter. As more and more adenovirus types are isolated from





human and animal sources it is probable that the suggested criteria will have to be modified. Andrewes et al (1961) suggest that (according to current concepts) for an agent to be classified as a member of the adenovirus group, it should have the following characteristics:

- 1) the basic infectious unit is a particle about 70 mu. in diameter composed of an inner core and an outer covering apparently made up of 252 sub-units (capsomeres) arranged to form an icosahedron;
- 2) it is resistant to inactivation by both ether and trypsin;
- 3) the infective particle is composed of protein and deoxy-ribonucleic acid but contains no ribonucleic acid (RNA);
- 4) it multiplies in the nucleus of infected cells and induces the formation of a specific protein which in turn causes characteristic CPE in the cell culture;
- 5) multiplication within the cell results in the formation of a soluble complement-fixing antigen; (this antigen crosses, immunologically, at least partially with similar antigens from other members of the adenovirus group) and
- 6) it is frequently the cause of inapparent infections of susceptible animal species and, following infections, persistence of the virus in a latent form is common.

Plate #1 is an electronmicrograph of the canine adenovirus Toronto A26/61 prepared using negative-contrast-staining procedures which involved the use of phosphotungstic





acid. The magnification is approximately 400,000 times actual size. Preparation and photography were done by Dr. T. Yamamoto.

Pereira et al (1963) divided the adenovirus group into subgroups based on natural hosts.

Hirst (1941) noticed that influenza virus group A caused the agglutination of chicken embryo erythrocytes and that specific antiserum against the virus would inhibit such hemagglutination. Hirst (1942) also discovered that there was a direct, quantitative relationship between the agglutinating capacity of influenza virus suspensions and their infectivity for mice, as well as between the agglutination-inhibition titre and the virus-neutralization titre of a serum.

Rosen (1958) conducted similar experiments on the hemagglutination phenomenon exhibited by human adenoviruses 1 to 18 against erythrocytes from different animal species. Human, Rhesus monkey, Grivet monkey, mouse, and rat erythrocytes were employed. Mouse and rat erythrocytes displayed the highest hemagglutination titre with the majority of the human adenoviruses examined. Specificity of the hemagglutination phenomenon by prototype strains was demonstrated by hemagglutination-inhibition tests using rabbit hyper-immune serum. Subsequently, Rosen (1960) showed that the hemagglutination-inhibition technique was suitable for the identifica-



tion of the vast majority of adenoviruses and that this technique was much simpler and less time consuming than the neutralization procedure. Rosen developed a hemagglutination-inhibition test which was used to identify 417 adenovirus isolates of 24 different types.

Fastier (1957) related that, under specific conditions of temperature and pH, the virus of infectious canine hepatitis enters into a reversible association with fowl erythrocytes. By adsorption with these cells both the hemagglutinin and the infective particle could be removed from tissue culture fluids containing ICH virus without appreciably reducing their ability to act as efficient complement-fixing antigens.

Cabasso (1962) in a review article on infectious canine hepatitis virus, reported that Kunishige and Hirato (1960) found that under certain conditions two different virus isolates could agglutinate human and guinea pig erythrocytes, but not those from sheep, rabbits, domestic fowl, horses, cows, dogs, or mice. Heat destroyed the hemagglutinin somewhat more slowly than the viral infectivity, but faster than the complement-fixing antigen. Removal of the hemagglutinin from infective fluids by adsorption with erythrocytes from a guinea pig or human resulted in a concomitant decrease in infectivity without materially affecting the complement-fixing antigen titre.



Kapsenberg (1959) has compared infectious canine hepatitis virus with several human adenoviruses on the basis of cytopathic effects, (ICH virus and primary dog kidney epithelial cells as compared with human adenovirus and human line cells) animal infection, and complement-fixation, and considers the virus of infectious canine hepatitis to be a member of the adenovirus group.

In addition to the gross changes in the appearance of the architecture of the cell monolayer due to adenovirus infection there are specific nuclear alterations in the individual infected cells. A number of authors, particularly Dingle and Ginsberg (1959) have studied the progression of these changes in the nuclei. They state that while not all of the known serotypes have been characterized by the changes they induce in the nucleus of infected cells, those that have been studied can be assigned to one or the other of two sub-families represented in one case by types 1, 2, 5 and 6 and in the other case by 3, 4 and 7.

In cell cultures infected with types 1, 2, 5 or 6 adenovirus characteristic changes appear in the nucleus 15 hours following infection. First, a number of eosinophilic inclusions appear. Then, as infection progresses, the eosinophilic inclusions develop basophilic cores and become less definite in outline. They eventually merge into a dense basophilic mass within the enlarged nucleus. In contrast, the





first changes associated with infection by types 3, 4 or 7 adenovirus strains are detected about 14 hours after infection. The changes consist of the formation of irregular, granular, eosinophilic masses, the development of a rarified zone beneath the nuclear membrane, and rearrangement of the chromatin into lattice or network patterns. Early in the course of infection well defined intranuclear eosinophilic bodies appear which are similar to those observed in cells infected with types 1, 2, 5 and 6 viruses. The nucleus becomes large and distorted. The clear peripheral zone of the nucleus widens and the central area becomes more intensely basophilic. Sharp-edged crystal-like masses appear in the affected nuclei. These masses display varying degrees of eosiniphilia and basophilia. In the latest stages of infection the central basophilic zone becomes more prominent. In some cells there are ovoid or wedge-shaped compartments radiating out from a central mass producing a flower-like nuclear form.

Kjellen et al (1955) were the first to demonstrate that the inclusions are composed of clusters of apparently mature virus particles in a crystalline array.

The properties of infectivity and self-replication seem to be carried by a complex, organized particle approximately 70  $\mu$ . in diameter. Commonly, infectivity of a preparation is measured by testing decremental dilutions of





the material in an appropriate number of sensitive cell cultures. Infected cultures are recognized by evidence of characteristic CPE through microscopic examination of the cell monolayers. From these data the  $TCID_{50}$  can be calculated by any of the standard procedures such as that of Reed and Muench (1938). In the case of adenovirus, early cytopathic effects can be caused by a soluble protein elaborated by infected cells (Rowe et al, 1958b), (Pereira, 1958), (Everett and Ginsberg, 1958), and (Pereira, et al 1959). Therefore, titrations based upon CPE which occur early in the growth cycle, do not measure the infectious capacity of the virus, but rather the amount of CPE-inducing, soluble protein. However, cultures held for periods long enough to allow recovery from the early CPE give a true estimate of the virus preparation.

It was originally thought that as many as a million or more virus particles were required for a single tissue culture infectious dose. This opinion was based on virus titrations read after three to seven days of incubation. However, experiments by Pereira and Valentine (1958) have shown that as few as ten characteristic particles comprise an infectious dose. Yamamoto (in press) has found in the case of canine adenovirus Toronto A26/61 that approximately 10,000 to 100,000 particles constitute an infectious dose.

The plaque technique for the titration of animal



viruses was introduced by Dulbecco (1952) and represents an important advance upon earlier more cumbersome methods of virus assay. The principle of the technique is that a monolayer of cells is infected with virus and after a suitable incubation period is overlaid with a nutrient agar. The agar prevents the products of virus multiplication from spreading throughout the medium as they would in a fluid culture. In this virus assay system, particles released from the initially infected cell enter adjacent cells where they undergo a further cycle of multiplication. The process continues until the focus of infected and damaged cells is large enough to be distinguished by the naked eye as an unstained area or "plaque" set in a background of healthy cells which take up a vital dye, commonly neutral red.

Plaque production by adenovirus was investigated by Bonifas and Schlesinger (1959) and by Kjellen (1961) on a variety of clonal cell lines. The difficulty encountered in trying to produce plaques was ascribed to a virus-induced nutritional deficiency. Each group found that a tenfold increase in the amount of arginine added to the Eagle's basic medium lead to a more rapid destruction of KB cells (a cell line derived from human epidermoid carcinoma) by human adenovirus type 2. When the virus-infected KB cell cultures were overlaid with agar containing Eagle's basic medium with increased concentrations of arginine (a four-





to ten-fold increase) the plaques appeared within nine to ten days. Neutral red was found to inhibit plaque formation when added to the first overlay. This problem was solved by adding the neutral red as a second agar overlay following the incubation period. The concentration of neutral red in the second 5.0 ml. agar overlay was 1:11,250. In a later paper Rouse et al (1963) verified the fact that established human cell lines were often contaminated with pleuropneumonia-like organisms (PPL0) and that these organisms have the capacity of rapidly depleting the available arginine, thereby lowering the efficiency of plating of various adenoviruses. The fluctuations in plating efficiency can be eliminated by curing the cells of PPL0 infection with Kanamycin (40 mg./ml. tissue culture medium applied for two days).

Inhibition of multiplications of pneumonia virus of mice (Horsfall and McCarty, 1947) and mumps (Ginsberg et al, 1948) can be accomplished with type-specific capsular polysaccharides of Friedlander bacilli. Furthermore, an algal polysaccharide was found to inhibit the growth of Lee strain of influenza virus (Gerber and Adams, 1958; Gerber et al, 1958).

Araki (1959) has shown that agar is a complex substance consisting of at least two polysaccharides, agarose and agaropectin. Agarose appears to have a linear





structure made up of alternating residues of  $\beta$ -D galactopyranose and 3, 6, anhydro L-galactopyranose and contains few if any charged groups. The structure of agarpectin is not well understood and it may possibly be a mixture of several polysaccharides. As a generalization, agarpectin differs from agarose in that it is sulfated and contains, in addition to the constituents of agarose, D-glucuronic acid and pyruvic acid. The inhibitory effect of agar on viral growth and plaque development appears to be due to the negatively charged groups which the agar contains, or which is found in commercial agar preparations.

Takemoto and Liebhaver (1961) found that wild-type encephalomyocarditis (EMC) virus produced minute plaques (1 mm. at four days) on L-cell monolayers with an occasional mutant which produced large plaques (8 - 10 mm. at four days). This phenomenon was ascribed to the sulfated polysaccharide present in the agar which inhibited the growth of the minute plaque former, but which had no effect on the large plaque mutant. Also, in the absence of the agar polysaccharide, the minute plaque former actually multiplied more rapidly than the mutant.

Liebhaver and Takemoto (1961) discovered that this inhibitor (a polyanionic sulfated polysaccharide released from the agar under autoclaving conditions) could be bound by a polybasic water-soluble molecule diethylaminoethyl



dextran (DEAE-dextran), removing its inhibitory effects from the agar overlay and thus increasing plaque size. Another polybasic compound, protamine sulfate, was also shown to bind the sulfated polysaccharide.

Miles and Austin (1963) found that application of DEAE-dextran and protamine sulfate to a number of arbovirus systems resulted in the production of clear-cut plaques to high titres.

Liebhaber and Takemoto (1963) ascribe the inhibition produced by the sulfated polysaccharide to an ionic interaction between the sulfated polysaccharide and the (r+) wild-type virus (but not the large plaque (r) variant) thus preventing the proper adsorption of the (r+) virus particle to the mammalian cell.

In the case of Mengo encephalomyelitis virus and its interaction with L-cells, Colter et al (1964) presented data that strongly supports the hypothesis that the agar inhibitor blocks cell-virus interaction by reacting directly with the virus particles most likely at those sites through which virus particles attach to cells. It was also reported that protamine itself was an inhibitor of virus-cell interaction in suspended cultures and it was suggested that protamine acts by reacting with the cell thus presumably blocking receptor sites on the cell surface. However, the possibility of the involvement of protamine intracellularly was not ruled out.





Campbell and Colter (1965) examining the effect of a number of different overlays on the sizes of plaques produced by three variants of Mengo encephalomyelitis virus (S, M, and L) have shown that certain anionic polymers (dextran sulfate, heparin, chondroitin sulfate) have little or no effect on the sizes of plaques produced in L-cell (C3H mouse fibroblast) monolayers by L- and S-Mengo variants but that M-Mengo virus plaque size is greatly affected. The plaque size may be either decreased or increased by individual polyanions. They suggest that molecular weight is a factor in determining the ability of a sulfated polysaccharide to enhance M-Mengo virus plaque size. Lower molecular weight preparations of sulfated hyaluronic acid enhance plaque size more efficiently than do higher molecular weight ones containing the same amount of sulfate. They postulate that lower molecular weight polymers may penetrate the cell efficiently and that they have some intracellular action resulting in a faster spread of virus in the cell monolayer.

Meselson et al (1957) developed a method for the study of the molecular weight and partial specific volume of macromolecules, viruses, and particulate materials. The method involves the observation of the equilibrium distribution of macromolecular material in a density gradient itself at equilibrium. The density gradient is established by the



sedimentation of a solute of appropriate molecular weight in a solution subject to a constant centrifugal field. The opposing tendencies of sedimentation and diffusion produce a stable concentration gradient. The concentration gradient and compression of the liquid result in a continuously increasing density along the direction of centrifugal force. In considering the distribution of a small amount of a single macrospecies in this density gradient the initial concentration of the solute, the centrifugal field strength, and the length of the liquid column may be chosen so that the range of density at equilibrium encompasses the effective density of the macromolecular material. The centrifugal force tends to drive the macromolecules into a region where the sum of the forces acting on a given molecule is zero. The effective density of the macromolecular material is defined as the density of the solution in this region. This concentrating tendency is opposed by Brownian motion with the result that at equilibrium the macromolecules are distributed with respect to concentration in a band of width inversely related to their molecular weight.

Sueoka et al (1959) demonstrated that the buoyant-density of DNA in cesium chloride was directly proportional to its (G + C) content. It was also shown that denatured DNA behaved in a similar manner but was uniformly higher in density.





Schildkraut et al (1962) did a comprehensive study of the buoyant-densities of DNA as a function of composition. A linear relationship was confirmed where buoyant-density ( $\rho$ ) =  $1.660 + 0.098 (G + C)$ . ( $G + C$ ) equals the mole fraction % ( $G + C$ ). It was also shown that DNA of the commonly studied T-even bacteriophages exhibited altered densities due to the presence of glucosylated hydroxymethyl cytosine.

Green and Pina (1963b) showed that the DNA's of non-tumorigenic adenoviruses types 2 and 4 have a mole fraction % ( $G + C$ ) of 56% to 57%, while DNA's of the tumorigenic adenoviruses types 12 and 18 have a mole fraction % ( $G + C$ ) of 48% to 49%. Thus, adenoviruses types 12 and 18 both have a % ( $G + C$ ) content quite different from the types 2 and 4 adenoviruses and closer to that of mammalian cell DNA which has a mole fraction % ( $G + C$ ) of 42% to 44% (Chargaff and Davidson, 1955).

The four tumor-inducing DNA-containing mammalian viruses, namely adenoviruses 12 and 18, polyoma virus and Shope papilloma virus, have very similar buoyant-densities and therefore base compositions. This similarity has led Green and Pina (1963b) to propose that it is possible that tumorigenic viruses have evolved from non-tumorigenic viruses by deletion of a piece of DNA rich in guanine and cytosine.

Kay et al (1952) used an anionic detergent (sodium lauryl sulfate) to precipitate the protein substituents of



calf thymus cells. The method has the advantage that the anionic detergent both dissociates the nucleoprotein complex into nucleic acid and protein, and also precipitates the protein with little or no fractionation of the nucleic acid.

The amount of protein in a preparation was reduced by the sequential use of the anionic detergent until little protein precipitate could be observed. The nucleic acid was then precipitated by ethanol.

The feasibility of separating nucleic acid derivatives by paper chromatography was demonstrated by Vischer and Chargaff (1948) and by Hotchkiss (1948). A method for the quantitative estimation of derivatives from ribonucleic acid was developed by Vischer and Chargaff (1948) and was later applied to deoxyribonucleic acids.

Chargaff et al (1949), Wyatt (1951) described a method where deoxyribonucleic acid is hydrolysed by formic acid to purines and pyrimidines which are then separated by paper chromatography. Marshak and Vogel (1950) reported quantitative hydrolysis of both RNA and DNA by 12 N-perchloric acid at 100°C for one hour without prior isolation of the nucleic acids.

Green and Pina (1963a), using perchloric acid hydrolysis, give the base ratio of human adenovirus type 2 as 27: 29: 22: 21; human adenovirus type 4 as 27: 30: 22: 20; KB tissue culture cells as 21: 21: 28: 30 for the purine and pyrimidine bases guanine, cytosine, adenine, and thymine, respectively.





## MATERIALS AND METHODS

### Virus Strains

The viruses used in these experiments were obtained from different sources. Toronto A26/61 virus was obtained from Dr. J. Ditchfield, University of Toronto. The infectious canine hepatitis virus was obtained from Dr. V. J. Cabasso, Lederle Laboratories, Pearl River, New York. The other strain of infectious canine hepatitis virus (hepatitis contagiosum canis - HCC) was obtained from Dr. J. G. Kapsenberg, Rijks Instituut Voor de Volksgezondheid Sterrenbos, 1 Utrecht, Netherlands.

### Tissue Culture Cells

Primary dog kidney epithelial cells prepared from young dogs two to six weeks old were used initially for the propagation of the three virus strains. Experimentation indicates that virus preparations could be prepared with similar levels of infectivity by infecting a continuous epithelial dog kidney cell line (#32690) obtained from Dr. R. C. Parker, Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

### Tissue Culture Media (see Appendices #1 to #5)

The following are the different types of tissue culture media used:

- 1) Hanks' lactalbumin medium (HLA). It consists of Hanks' balanced salt solution (Hanks and Wallace, 1949) with 0.5% lactalbumin hydrolysate (DIFCO 0996-01). HLA





medium was used for the initial propagation of primary dog kidney epithelial cells to the monolayer stage.

- 2) Earle's lactalbumin with yeast extract (ELY). It consists of Earle's balanced salt solution (Earle et al, 1943) with 0.5% lactalbumin hydrolysate (DIFCO 0996-01) and 0.1% yeast extract (DIFCO 0127-01). ELY medium was used to maintain the secondary PDK cells after subcultures had been made of the original monolayer.
- 3) Eagle's minimal essential medium (Eagle, 1955) combined with Hanks' balanced salt solution (EHB). This medium contains only l-amino acids (MEM Eagle essential amino acids 50 x concentrate #71-059R Baltimore Biological Laboratories, Baltimore, Maryland). Because of instability even at refrigerator temperatures, glutamine is not included in the original solution of l-amino acids and was added prior to use in the concentration of 292 mg. of glutamine per litre of medium. EHB medium was used for the nutrition of DKL cells during studies of plaque assay methods.
- 4) Eagle's minimal essential medium with Earle's balanced salt solution (EEB). This medium was used for the nutrition of DKL cells during studies of plaque assay methods. The medium was also the medium of choice for the propagation of viruses in DKL cell monolayers.



## 5) Medium 199 with Hanks' balanced salt solution (199).

This medium is prepared according to the formula of Morgan et al, 1950. It was used by Morgan et al, 1950 and Parker et al, 1957 to promote continuous cell multiplication in the absence of serum or embryo extracts. It contains d- and l-amino acids. Medium 199 was used for the nutrition of DKL cells during studies of plaque assay methods.

## 6) Earle's balanced salt solution with 1% proteose-peptone (DIFCO 0122-01) added (EPP). This medium was used as a nutritive medium for a plaque overlay using primary dog kidney cells.

7) Minimal essential medium (MEM). The medium is a recently introduced powdered medium produced by General Biochemicals, Laboratory Park, Chagrin Falls. The exact formula has not been reported to date. The first literature on the testing of this medium appears in a paper by Greene et al (1965). The designation of the medium is GBI Powdered Medium MEM (Eagle) with l-glutamine, without sodium bicarbonate, lot #651397.

MEM was used for the propagation of DKL cells.

### Propagation of Viruses

All of the canine adenovirus strains were propagated, routinely, in Roux bottles on monolayers of DKL cells containing approximately 30 million cells.



Prior to the inoculation of the monolayer with virus suspensions the nutritive medium was removed, and the cells were washed three times with Hanks' balanced salt solution. A suspension of virus in Hanks' balanced salt solution was added to the monolayer of cells (10 ml. of a  $7.5 \times 10^7$  TCID<sub>50</sub>/ml. suspension). The virus suspension was allowed to remain in contact with the DKL cell monolayer for two hours at 25°C with agitation every fifteen minutes to ensure complete coverage of the monolayer with the virus inoculum. At the end of the two hour adsorption time new medium (EEB containing 50 ug./ml. streptomycin and 50 i.u./ml. penicillin and 10% calf serum) was added to the bottles. The infected monolayers were incubated for five days at 37°C.

Besides the DKL cell line, primary dog kidney epithelial cells were also used for the propagation of Toronto A26/61 virus. The nutritive medium used for the propagation of PDK cells to the monolayer stage was HLA with 50 i.u./ml. penicillin and 50 ug./ml. streptomycin plus 10% calf serum. Maintenance of PDK cells was with ELY medium containing 50 i.u./ml. penicillin and 50 ug./ml. streptomycin with 10% calf serum.

#### HEMAGGLUTINATION AND HEMAGGLUTINATION-INHIBITION PROCEDURES

##### Hemagglutination Test

The hemagglutination test was performed by preparing







serial doubling dilutions of hemagglutinin to each of which was added a standard amount of human erythrocytes. The method was as follows: 0.25 ml. of physiological saline was added to a series of 13 x 100 mm. test tubes with the aid of the Cornwall automatic pipette. A 0.5 ml. suspension of the virus to be tested was added to an empty test tube (tube #1); 0.25 ml. of this 0.5 ml. suspension was transferred to tube #2 containing 0.25 ml. of physiological saline. This dilution (by one-half) in tube #2 was mixed by pipetting up and down three times. Then 0.25 ml. was transferred to tube #3 creating a dilution this time of one-quarter. This procedure was continued until twelve tubes of dilutions had been prepared. The last tube exhibited a dilution of 1/4096 and the sequence of the dilutions was 1/2, 1/4, 1/8, 1/16, and so on to the final dilution of 1/4096.

The erythrocytes for the hemagglutination test were obtained as citrated blood from the Red Cross Transfusion Service. The erythrocytes were sedimented by centrifugation at 2,000 rpm. for fifteen minutes and washed three times with physiological saline. The red cells were resuspended in Alsever's solution (Kalter, 1963) (see Appendix #7) as a 0.5% suspension. 0.25 ml. of the 0.5% suspension of erythrocytes was added to each tube containing the virus dilutions. The tubes were shaken until a uniform



dispersion of erythrocytes was observed. The hemagglutination test was incubated at 25°C for one hour. The hemagglutination unit for influenza virus has been defined as:

- 1) the highest dilution of virus in 0.5 ml. volume causing complete agglutination of chicken erythrocytes contained in 0.5 ml. of a 0.5% suspension (Committee on Serological Procedures in Influenza Studies, 1950 and W.H.O. Technical Report series No. 64, 1953); and
- 2) the highest dilution of influenza virus in a 0.25 ml. volume causing partial (50%) agglutination of chicken erythrocytes contained in 0.25 ml. of a 0.5% suspension (W.H.O. Technical Report Series No. 64, 1953).

The final hemagglutination readings were taken after overnight incubation at 25°C. By analogy with influenza virus the hemagglutination unit for adenovirus was the highest dilution of the three strains of virus tested in 0.5 ml. volume causing complete agglutination of human erythrocytes contained in 0.5 ml. of a 0.5% suspension.

#### Hemagglutination-Inhibition Test

The method for the testing of hemagglutination-inhibition was as follows:

A series of 100 x 13 mm. test tubes was placed in a holder; 0.25 ml. of physiological saline was added to each tube. The first tube in the series contained only 0.5 ml. of specific rabbit antiserum. 0.25 ml. of this antiserum



was added to tube #2 which contained 0.25 ml. of physiological saline. The dilution of antiserum in tube #2 was therefore two-fold. This mixture was pipetted up and down three times and 0.25 ml. was transferred to tube #3. In this manner doubling dilutions of the antiserum in saline were completed to 20 tubes.

To each tube was added 0.25 ml. of the virus suspension to be tested. The mixture was shaken and allowed to incubate for 30 minutes at room temperature (25°C). These incubation conditions allowed a combination to take place between the virus antigen and antibody prior to the introduction of 0.25 ml. of a 0.5% suspension of erythrocytes which was added to each tube of the system and the series was incubated at room temperature for one hour. Readings for hemagglutination-inhibition were recorded. The end point was taken as the first dilution tube to show agglutination of the erythrocytes. The tubes were then incubated overnight and any changes in the original readings were recorded.

#### CYTOPATHOLOGY

Dog kidney cells were propagated in Leighton tubes on 5 x 22 mm. glass slides until monolayers had formed (approximately  $6 \times 10^5$  cells). The monolayer cell sheets were inoculated with a virus suspension of known titre and the cells were incubated for 72 hours. The procedure was







as follows: DKL cells were suspended to a density of 250,000 cells per ml. in EEB containing 50 i.u./ml. penicillin and 50 ug./ml. streptomycin plus 10% calf serum. 2.0 ml. aliquots were dispensed into Leighton tubes containing 5 x 22 mm. glass cover slips. The tubes were stoppered with white latex rubber stoppers, placed in a Leighton tube rack and were incubated at 37°C until a complete monolayer had formed. 0.1 ml. of a suspension of Toronto A26/61 virus exhibiting an infectivity of  $3.3 \times 10^6$  PFU/ml. was inoculated into 40 tubes. Ten tubes (uninfected) were kept as controls. The infected and uninfected monolayers were incubated at 37°C. Every six hours, four infected monolayers and one uninfected monolayer were removed and were fixed with Zenker's fixative (Humanson, 1962). Since the procedures for fixing and staining described by Humanson (1962) are for gross tissue sections, a modified procedure was developed and is detailed below. (The different ingredients which make up the staining materials appear in Appendix #8). The method of treatment of the coverslips is as follows:

- 1) Fix in Zenker's fixative 30 minutes.
- 2) Place in Lugol's iodine until brown (30 seconds).
- 3) Rinse in tap water and place in a 5% solution of sodium thiosulfate for 30 seconds until the brown color disappears.



- 4) Rinse in tap water and stain in hematoxylin for 30 seconds.
- 5) Rinse in tap water and then rinse in Scott's solution until the tissue changes from a pinkish hue to a blue hue.
- 6) Wash in tap water and place in water-eosin for two minutes.
- 7) Dehydrate by rinsing quickly through ethanol-eosin, 70% ethanol, 95% ethanol and then absolute ethanol.
- 8) Place in xylene for two minutes.
- 9) Mount on an ordinary glass slide using Permount (Fisher Scientific Co.).

This method causes the nuclei and basophilic inclusions to appear dark blue and the cytoplasm and nucleoplasm to appear pink. Strongly eosinophilic inclusions appear bright red.

Photomicrographs were taken of normal uninfected dog kidney cells and of infected dog kidney cells.

The same staining procedures were used employing Toronto A26/61 virus-infected PDK cells.

#### VIRUS ASSAY

Two methods of virus assay were examined:

- 1) A method which determines the 50% tissue culture infectious dose (TCID<sub>50</sub>).
- 2) Determination of virus titre in terms of plaque-forming



units (PFU).

#### Tissue Culture Infectious Dose 50% Method

The protocol used to assay virus suspensions by the TCID<sub>50</sub> method was as follows:

2.0 ml. of DKL cells in a concentration of 250,000 cells/ml. were inoculated into 15 x 125 mm. etched test tubes. The tubes were stoppered with number 0 white latex rubber stoppers. These tubes were incubated at 37°C at an angle of about 10° to the horizontal. The cells settle to the lower surface of the tube opposite the etched label. They grow into a monolayer to the extent of the tissue culture medium.

Serial ten-fold dilutions were made of the virus to be assayed, by adding 0.2 ml. of a virus suspension to a 15 x 125 mm. tube containing 1.8 ml. of Hanks' balanced salt solution. This suspension was pipetted up and down three times and 0.2 ml. of this suspension was transferred to the next tube in the series until ten-fold dilutions ranging from  $1 \times 10^{-1}$  to  $1 \times 10^{-8}$  were completed. 0.1 ml. of the viral dilutions were added to the titration tubes. The titrations were done in triplicate. These tubes were incubated from ten to sixteen days and were read for cytopathic effect every day until no further change was observed. Controls were kept which had 0.1 ml. of Hanks' balanced salt solution added instead of the virus suspension. If







the pH dropped significantly due to increased cellular metabolism, fresh medium was added in place of the old.

### Plaque-Forming Unit Method

One of the problems to be solved during the course of this research project was to produce a satisfactory method for the assay of Toronto A26/61 virus by the plaque method.

The protocol for the preparation of materials for the plaque assay of Toronto A26/61 is essentially the same for each medium tested. Hence, it will serve brevity to give a general account of the method used.

Double strength Hanks' or Earle's balanced salt solutions were prepared. The balanced salt solutions were sterilized by autoclaving for 15 minutes at 121°C and 15 psi. pressure. Vitamins, amino acids, calf serum, and antibiotics were added (aseptically) so that the concentrations were twice the concentrations desired in the final agar overlay, since equal volumes of special Noble agar (DIFCO 0142-02) were required to be added, prior to the addition of the nutritive overlay to the infected monolayer of cells. Two types of tissue culture vessels were used during the plaque assay studies: (1) 3 oz. prescription bottles stoppered with white latex rubber stoppers, size 0; and (2) 15 x 60 mm. sterile disposable tissue culture dishes (TCD 3002 Falcon Plastics). Several different types



of media were examined in an endeavor to find the most reliable medium for the reproducibility of results for the assay of Toronto A26/61 virus using the plaque assay method. Different additives known to enhance the formation of plaques in other cell-virus systems (Liebhaber and Takemoto, 1961, Takemoto and Liebhaber, 1961, Liebhaber and Takemoto, 1963, Miles and Austin, 1963, Colter et al, 1964, Campbell and Colter, 1965) were examined. These are: (1) protamine sulfate (salmine) Mann Research Laboratory #1451; and (2) DEAE-dextran (Pharmacia Uppsala Sweden, #72).

Tissue culture dishes containing monolayers of DKL cells infected with Toronto A26/61 virus were incubated in a 5% CO<sub>2</sub>, 95% filtered air atmosphere in a National Incubator (Model 350T, National Appliance Co., Portland, Oregon) at 37°C. Three-ounce prescription bottles containing monolayers of DKL cells infected with Toronto A26/61 virus were incubated at 37°C in a standard walk-in incubator.

Penicillin and streptomycin were added to all media in a concentration of 50 i.u./ml. and 50 ug./ml. respectively. Because the methods used in the following experiments are peculiar to each experiment, they are related under appropriate headings in the "RESULTS" section. The headings are:

Plaque Assay Using Primary Dog Kidney Cells

Plaque Assay Using Dog Kidney Line Cells



Effect of Varying Concentrations of Two Chemical Additives  
on Plaque Formation

CELL DENSITY AND CONCENTRATION OF VIRUS INOCULUM STUDIES

CONCENTRATION OF TORONTO A26/61 VIRUS

BUOYANT-DENSITY STUDIES

Buoyant-Density Comparison Between Toronto A26/61

and ICH Viruses

The Relationship Between the Buoyant-Density of Deoxyribo-  
Nucleic Acid and the Mole Fraction % (G + C)

Because analytical amounts of DNA could not be extracted from concentrated preparations of Toronto A26/61 virus, it was decided to extract Toronto A26/61 virus-infected DKL cells by the method of Kay et al (1952) and submit the extracted DNA to gradient centrifugation to equilibrium in the hope that if there were two species of DNA (one viral and one host cell) these could be separated into two bands because of some difference in the mole fraction % (G + C). Meselson et al (1957), Sueoka et al (1959), Schildkraut et al (1962).

Thirty, ten-ounce prescription bottles were seeded with 1.0 ml. of DKL cells at a density of 250,000 cells/ml. and were allowed to propagate to monolayers in a nutritive medium (EEB plus 10% calf serum). The monolayers were then washed in Hanks' balanced salt solution, and inoculated with a suspension of Toronto A26/61 virus at a concentration of







approximately 25 TCID<sub>50</sub>/cell. The virus suspension was allowed to adsorb to the monolayers for two hours at 25°C with agitation every fifteen minutes to ensure complete coverage of the monolayers with the virus inoculum. After adsorption fresh medium was added and the infected monolayers were incubated at 37°C until complete cytopathic effect was observed. The cell sheets were then removed (using a rubber policeman) into the nutritive medium. Cells were then sedimented by centrifugation. Control, uninfected cells of DKL cells were also harvested.

DNA was extracted from the infected and uninfected cells using a modification of the method described by Kay et al (1952). (The procedure appears under the "CHEMICAL STUDIES" section.) 300 ug. of DNA from infected and non-infected cells were added to 5.0 ml. cellulose nitrate centrifuge tubes containing an 89% aqueous solution ( $\rho = 1.778$ ) of CsCl.

To test the reliability of the technique, 0.5 ml. of a 1.0 mg./ml. solution of grade A salmon sperm DNA (of known mole fraction % (G + C) and buoyant-density) was added to a third 5.0 ml. cellulose nitrate centrifuge tube. The solution was evaporated to dryness in vacuo over phosphorus pentoxide. The dried DNA was dissolved in 5.0 ml. of an 89% aqueous CsCl solution. The three tubes, each containing DNA, were placed in the type #39 rotor of the Beckman model



L-2 preparative ultracentrifuge and were centrifuged at 35,000 rpm. for 48 hours.

At the termination of the centrifugation, holes were punched into the bottom of each centrifuge tube using a 20 gauge hypodermic needle and five-drop fractions were collected. The density of each fraction was calculated and recorded. Subsequently, 3.0 ml. of 0.1 N HCl were added to each fraction and each was scanned spectrophotometrically (between 200 m $\mu$ . and 340 m $\mu$ .) in the Bausch and Lomb #505 recording spectrophotometer. The occurrence of any fraction exhibiting absorbance typical of DNA was correlated with the density of the fraction in which it was found.

#### CHEMICAL STUDIES

Base ratio analyses were attempted on DKL-cell DNA and Toronto A26/61 virus DNA. Grade A salmon sperm DNA (for which the base ratios have been determined, Chargaff et al, 1951) was used to test both the reliability of the acid hydrolysis as well as paper chromatographic separation of the products of hydrolysis. DNA absorbance curves were constructed using salmon sperm DNA and DKL-cell DNA. Also, absorbance curves were constructed using purine and pyrimidine bases derived from the acid hydrolysis of salmon sperm DNA, DKL-cell DNA and Toronto A26/61 virus DNA. In addition, absorbance curves were constructed using grade A commercial preparations of the individual purine and pyrimidine bases



guanine, cytosine, adenine, thymine, and uracil. Sample chromatograms were constructed locating purine and pyrimidine bases in their relative positions as they would appear after chromatography. Bases derived from salmon sperm DNA, DKL-cell DNA, Toronto A26/61 virus DNA, and individual purine and pyrimidine bases from commercial sources were examined.

#### Extraction of DNA from DKL Cells

The method used for the extraction of DNA from DKL cells was a modification of a method described by Kay et al (1952). DKL-cells were propagated to the monolayer stage in Roux bottles. Approximately one gram of DKL cells harvested from ten Roux bottles were collected after centrifugation in the International Model SB centrifuge (using #259 head) at 2,000 rpm. for fifteen minutes. The cells were washed three times with 0.9% sodium chloride and 0.01 M. citrate solution and were then sedimented into a pellet in a 15 ml. screw cap centrifuge tube on the Cave Desk centrifuge at 1,000 rpm. for fifteen minutes. One gram of DKL cells was suspended in 20 ml. of 0.9% sodium chloride and 0.01 M. citrate. 2 ml. of 5% sodium lauryl sulfate in 45% ethanol were added. The suspension was stirred for twenty minutes at room temperature until viscous and 1.6 grams of NaCl was added to give a concentration of 1.0 M. The solution was stirred until the sodium chloride had dissolved. This caused the precipitation of protein and







ribonucleic acid concurrent with a decrease in viscosity of the solution. The suspension was centrifuged at 2,500 rpm., at 5°C using the SS34 rotor in the Sorvall RC2 centrifuge, for ten minutes. The supernatant was retained and one volume of 95% ethanol was added. A glass stirring rod was admitted to the test tube and the alcohol, which was the top phase of the two-phase system, was slowly stirred into the 0.9% sodium chloride and 0.01 M. citrate phase. A gelatinous mass was wound onto the stirring rod which turned to a fibrous mass after washing three times in 95% ethanol and several times in acetone until the acetone was no longer cloudy. The fibrous mass was redissolved in 14 ml. of distilled water and 1.25 ml. of sodium lauryl sulfate in 45% ethanol was added. The solution was stirred at room temperature for twenty minutes. The solution was brought to 1.0 M. sodium chloride by the addition of 0.81 grams of sodium chloride. The solution was centrifuged at 13,000 rpm. for one hour (using the type SS34 rotor in the Sorvall RC2 centrifuge). The supernatant was retained and one volume of 95% ethanol was added with stirring. A gelatinous mass again appeared on the stirring rod which was subsequently washed three times in 95% ethanol and several times in acetone. This fibrous residue was again redissolved in 14 ml. of distilled water. The solution was brought to 0.9% sodium chloride by the addition of 0.137 grams of



sodium chloride. The solution was centrifuged at 13,000 rpm. for one hour (using the type SS34 rotor in the Sorvall RC2 centrifuge). The supernatant was retained and was brought to 1.0 M. NaCl by the addition of 0.784 grams of sodium chloride. The sodium deoxypentose nucleate was precipitated by the careful addition of one volume of 95% ethanol with stirring. The fibrous preparation was redissolved in 3 ml. of 0.1 M. NaCl and 0.1 M. Tris buffer pH 7.0, and an absorbance curve (between 200 mu. and 340 mu.) of this DKL-DNA was made using the Bausch and Lomb #505 recording spectrophotometer. All DNA preparations were treated for eight hours with ribonuclease (RNase) at a concentration of 5 ug./ml. of DNA solution prior to chemical studies on the DNA. The DNA was reprecipitated from the treated solution and then resolubilized before examination.

#### Preparation of Salmon Sperm DNA

Grade A salmon sperm DNA was dissolved in 0.1 M. sodium chloride and 0.01 M. Tris buffer at pH 7.0 to a concentration of 1.0 mg./ml. From this solution a dilution was made to contain 50 ug./ml. This solution was subjected to a spectrophotometric analysis (between 200 mu. and 340 mu.) (see Figure #23)

#### Formic Acid Hydrolysis of DNA

Grade A salmon sperm DNA was used as a control to test the reliability of the formic acid hydrolysis technique





described by Wyatt, (1951). Hydrolysis of the deoxyribonucleates with the concurrent release of purine and pyrimidine bases was done in the following manner: 1.0 ml. of a 1.0 mg./ml. solution of salmon sperm DNA and 1.0 ml. of a 1.0 mg./ml. solution of DKL-cell DNA were placed in separate 13 x 100 mm. pyrex test tubes. The solutions were evaporated to dryness in vacuo over phosphorus pentoxide. 0.5 ml. of a 90% solution of formic acid was added to each tube containing the DNA and the tube was sealed in an oxygen-gas flame. The sealed tubes were placed in a wire test tube holder and were submerged in high-vacuum oil which was heated to 175°C over a Bunsen burner for 30 minutes (considered by Wyatt to be the optimal time of hydrolysis).

After hydrolysis the tubes were cooled and unsealed by holding the sealed tip of the tube in an oxygen-gas flame. The hydrolysates were evaporated to dryness in vacuo over phosphorus pentoxide in the same tubes. 1.0 N HCl was added to the tubes containing the dried hydrolysates of DNA in sufficient volume to make a solution of 2% to 4% (W/V) with respect to the weight of DNA hydrolyzed. The hydrolysates were allowed to dissolve and the solutions were applied to Whatman #1 chromatographic paper with a micro-pipette.

A reference chromatogram was prepared using the individual bases -- guanine, cytosine, adenine, thymine, and





uracil. Descending paper chromatography (Hotchkiss, 1948) was used for the separation of the bases as follows: after the hydrolysates had been spotted and dried onto the chromatographic paper, the papers were folded so that they could be held in a solvent trough by a glass rod. The length of the chromatograms hung down in a descending fashion from a glass support rod. A petri dish containing solvent (65% isopropanol V/V and 2.0 N HCl in the whole volume) was placed on the bottom of a sealed chromatographic tank containing the chromatograms. The chromatograms were allowed to equilibrate for one hour at 25°C, then solvent was placed in the chromatographic solvent troughs and the chromatograms left undisturbed for 43 to 46 hours. After this time period the chromatograms were dried overnight at 25°C. Contact photographic prints were prepared from each chromatogram using high contrast photographic paper and an ultra violet hand lamp (Markham and Smith, 1949). Drawings were subsequently prepared from the photographic prints locating the purine and pyrimidine bases in reference to one another and also in reference to the standard bases. Areas of each base on the chromatogram paper were cut from the chromatograms and the bases eluted from the paper (using 5.0 ml. of 0.1 N HCl) at 37°C for 12 hours. These eluents were quantitated spectrophotometrically and the base ratios were calculated from these data.



### Perchloric Acid Hydrolysis of Toronto A26/61 Virus

Several attempts to extract DNA from Toronto A26/61 virus failed. It was decided to hydrolyse the virus intact (Marshak and Vogel, 1950) and then attempt to elucidate the base ratios using the same paper chromatographic techniques used earlier for the separation of bases when pure samples of DNA were hydrolysed.

Toronto A26/61 virus was harvested from 15 Roux bottles of infected DKL cells. The virus was concentrated in a preformed CsCl density gradient. The preformed gradient was prepared as follows: solutions with different densities were prepared using CsCl and ion-free water.

<u>Solution #</u>	<u>H<sub>2</sub>O Added</u>	<u>CsCl Added</u>	<u>Calculated Density</u>
1	10 ml.	1.2 gms.	1.106
2	10 ml.	2.5 gms.	1.184
3	10 ml.	4.5 gms.	1.309
4	10 ml.	6.0 gms.	1.393
5	10 ml.	8.0 gms.	1.500

0.9 ml. of solution #5 was added to a 5.0 ml. cellulose nitrate centrifuge tube. 0.9 ml. of solution #4 was then layered very carefully upon solution #5. Then 0.9 ml. of solution #3 was layered upon solution #4, and so on, until all five solutions had been added to the centrifuge tubes. After very light stirring with a needle, the centrifuge tube therefore contained a preformed density gradient



ranging from 1.106 at the top of the tube to 1.500 at the bottom of the tube. Partially purified Toronto A26/61 virus (in a total volume of 0.5 ml.) was then added to the top layer (solution #1) of the tube, making a total volume in the centrifuge tube of 5.0 ml. The procedure was done in triplicate and the three tubes were placed in the type #39 rotor of the Beckman L-2 preparative ultracentrifuge. The tubes were centrifuged at 35,000 rpm. for six hours. At the completion of the centrifugation the centrifuge tubes were removed, holes were punched in the bottoms with a 20 gauge hypodermic needle and the contents of the centrifuge tubes were dripped into collecting tubes. Visibly obvious bands of virus were collected separately. The densities of the fractions (containing the bands) were determined experimentally. The fractions containing the bands were also subjected to spectrophotometric analysis (between 200 mu. and 340 mu.) to determine whether or not an absorbance curve typical of the Toronto A26/61 virus was evident.

Viral fractions from the three tubes (carried by the type #39 rotor of the Beckman L-2 preparative ultracentrifuge) were combined (total volume 3.0 ml.) and dialysed against one litre of 0.01 M. Tris buffer pH 8.0 at 5°C for 24 hours to remove the CsCl from the preparation. The liquid content of the dialysis tubing was then reduced to







zero by drying in a current of air in a fume cupboard at 25°C. The dialysis tube was then slit and the contents were washed into a centrifuge tube with 1.0 ml. of 0.01 M.  $\text{MgCl}_2$  in Tris buffer at pH 8.0. DNase and RNase were added to the virus suspension which was incubated at room temperature for eight hours. The virus was subsequently resedimented and dried in vacuo over phosphorus pentoxide in a 13 x 100 mm. pyrex tube. Perchloric acid hydrolysis (essentially the same method described by Green and Pina, 1963a) of Toronto A26/61 virus was attempted in the following manner: 0.5 ml. of perchloric acid was added to the pyrex tube containing the Toronto A26/61 virus. The tube was sealed with the aid of an oxygen-gas flame. The virus was hydrolysed (for one hour at 100°C) in a water bath, cooled, and the tube opened by melting the sealed tip. A solution of 3.95 M. KOH was added dropwise to the hydrolysate until neutral pH was achieved. This procedure caused the precipitation of potassium chlorate concurrent with the pH neutralization of the hydrolysate. The potassium chlorate was sedimented by centrifugation at 10,000 rpm. for ten minutes in the type SS34 rotor of the Sorvall RC2 centrifuge. The hydrolysate was then spotted directly onto Whatman #1 chromatographic paper using a micropipette. Descending chromatographic procedures for 46 hours were employed for the separation of bases using the same iso-



propanol - HCl solvent as previously described. Ultra violet absorbing regions representing the bases were located on the chromatogram using an ultra violet hand lamp. These areas were cut from the chromatogram and eluted with 3.0 ml. of 0.1 N HCl. The eluents were quantitated spectrophotometrically and the base ratios calculated from these data.

A composite drawing taken from chromatograms was prepared locating the separated bases of commercial grade A preparations of the individual bases, salmon sperm DNA, DKL-cell DNA, and Toronto A26/61 virus DNA (see Plate #7).



## RESULTS

### HEMAGGLUTINATION AND HEMAGGLUTINATION-INHIBITION PROCEDURES

#### Hemagglutination Test

Toronto A26/61 virus and the two strains of infectious canine hepatitis virus (ICH, HCC) were examined for their ability to cause hemagglutination using human erythrocytes from the blood groups, A, B, AB, and O. The results of these examinations appear in Table #1.

Table #1

<u>Virus Tested</u>	<u>Human Erythrocytes of Blood Group</u>			
	<u>A</u>	<u>B</u>	<u>AB</u>	<u>O</u>
A26	2048*	2048	2048	2048
ICH	1024	1024	1024	1024
HCC	2048	2048	2048	2048

\*Reciprocal hemagglutination titre -- a hemagglutination unit is defined as the highest dilution of virus in a 0.5 ml. volume causing complete agglutination of the erythrocytes contained in 0.5 ml. of a 0.5% suspension of erythrocytes.

Each virus examined entered into a hemagglutination reaction with erythrocytes from each of the human blood groups. Moreover, any one virus displayed equal hemagglutinating ability with each of the groups examined (i.e. Toronto A26/61 virus gave the same reciprocal hemagglutination





titre with erythrocytes from Group A, B, AB, or O). Only human erythrocytes were examined.

#### Hemagglutination-Inhibition Test

The results of the examination of Toronto A26/61 virus specific antisera (prepared by intravenous injection of a Toronto A26/61 virus suspension into two rabbits designated #1927 and #1950) for their ability to cause hemagglutination-inhibition in an agglutinating system using type "O" human erythrocytes and a suspension of Toronto A26/61 virus ( $TCID_{50} = 1 \times 10^6$ ) appear in Table #2.

Table #2

<u>Rabbit Antiserum Specific for Toronto A26/61 Virus</u>	<u>Reciprocal Hemagglutination Titre of the Antibody</u>
#1927	2048
#1950	4096

#### CYTOPATHOLOGY

The results of the inoculation of DKL cells and PDK cells with Toronto A26/61 virus are summarized in Table #3, page 43.



Table #3

<u>Hours Post Infection</u>	<u>Density of Intranuclear Inclusions</u> <u>DKL Cells</u>	<u>PDK Cells</u>
0	-	-
6	-	-
12	-	-
18	-	-
24	-	-
30	-	-
36	+	+
42	+	++
48	+	+++
72	+	+++

+ low number of inclusions (at 500 x, 1 or 2 nuclei with inclusions)

++ moderate number of inclusions (at 500 x, approximately 40% of nuclei with inclusions)

+++ larger number of inclusions (at 500 x, 90% of nuclei with inclusions and gross degeneration of cell monolayer)

Above densities based on the observation of several different fields.



Inclusions appeared in DKL cells and PDK cells at approximately the same time post infection (36 hours), but were relatively few in number in the case of DKL cells. The low number of intranuclear inclusions appearing in the DKL cells was ascribed to a relatively low multiplicity of infection (0.1 ml. of a Toronto A26/61 virus suspension exhibiting an infectivity of  $3.3 \times 10^6$  PFU/ml.\*, or approximately one PFU per two cells). Therefore, when PDK cells were inoculated, 1.0 ml. of the virus inoculum was used, or approximately five PFU per cell.

Photomicrographs were taken of normal and Toronto A26/61 virus-infected PDK cells and are presented in Plates 2, 3, 4, and 5.

Plate #2 is a photograph of an uninfected monolayer of cells. The nucleoli which appear as dark areas in the nucleus are a deep blue or basophilic color. The cytoplasm and nucleoplasm stains a pinkish or an eosinophilic color. One cell in the centre of the photograph is undergoing mitosis and the chromosomes are stained a dark blue.

Plate #3 is a photograph of an infected monolayer of PDK cells. Inclusions within the nucleus are obvious. Cell degeneration and pyknosis can be observed. This monolayer was photographed 48 hours post infection.

Plate #4 is a photograph of an infected monolayer at a higher magnification. Inclusions can be observed in

\*See Page 23





different stages of formation within different cells.

- (A) Indicates a nucleus in the early stages of inclusion formation.
- (B) Indicates a nucleus with the inclusions further along in development. The interiors of the inclusion, as viewed after hematoxylin-eosin staining, are a bright pink with basophilic borders becoming evident.
- (C), (D), and (E) Represent an even later stage. The eosinophilic centres of the inclusions are very faint in these nuclei. The vacuolation around the nucleus of (D) and (E) is characteristic of this stage.
- (F) Indicates complete basophilia of the nucleus. The eosinophilia which indicates the presence of protein is very faint and the basophilia which indicates the presence of nucleic acid is very prominent.

## VIRUS ASSAY

### Tissue Culture Infectious Dose 50% Method of Viral Assay

Below is the result of a tube titration of stock Toronto A26/61 virus propagated in DKL cells using as a nutritive medium EEB, 10% calf serum, 50 i.u./ml. penicillin and 50 ug./ml. streptomycin. The Reed and Muench (1938) method of calculation of TCID<sub>50</sub> is also shown.



Table #4

<u>Date</u> 1964	<u>Dilutions</u>								
<u>Sept.</u>									
25	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	c
28	+++	+++	---	---	---	---	---	---	---
30	+++	+++	+++	+++	---	---	---	---	---
<u>Oct.</u>									
1	+++	+++	+++	+++	+++	+++	---	---	---
5	+++	+++	+++	+++	+++	+++	---	---	---

Table #5

<u>Log Dilution</u> <u>of Virus</u>	<u>Obsv. No. of</u> <u>P(+) &amp; N(-) Cult.</u>		<u>Cum. No.</u> <u>Cult.</u>		<u>% Positive</u> $\frac{P}{N+P} \times 100$
	<u>P(+)</u>	<u>N(-)</u>	<u>P(+)</u>	<u>N(-)</u>	
1 x 10 <sup>-8</sup>	0	3	0	5	$\frac{0}{0+5} \times 100$ = 0%
1 x 10 <sup>-7</sup>	1	2	1	2	$\frac{1}{1+2} \times 100$ = 33.3%
1 x 10 <sup>-6</sup>	3	0	4	0	$\frac{4}{4+0} \times 100$ = 100%

Calculation of the logarithm of the 50% end-point dilution (ED<sub>50</sub>):

The 50% end-point lies between 1 x 10<sup>-6</sup> and 1 x 10<sup>-7</sup>.

$$\begin{aligned} \text{Log ED}_{50} &= -7.0 + (-6-7) \frac{33.3 - 50}{33.3 - 100} \\ &= -7 + 0.25 \\ &= -6.75 \end{aligned}$$

(NOTE: Exponent, base 10  
= logarithm)

Therefore, ED<sub>50</sub> = 10<sup>-6.75</sup>



Therefore, the titre of the virus suspension in terms of  $TCID_{50}$  is:  $1 \times 10^{6.75}/0.1$  ml. (because it is the reciprocal of the  $ED_{50}$  of 0.1 ml. of the virus suspension), or,  $1 \times 10^{7.75}/1.0$  ml. which is the infectivity per ml. of the original suspension of Toronto A26/61 virus.

#### Plaque Assay Using PDK Cells

Primary dog kidney cells were prepared as described in Appendix #6. They were suspended in HLA medium containing 50 i.u./ml. penicillin and 50 ug./ml. streptomycin plus 2% calf serum and inoculated into three-ounce prescription bottles in 10.0 ml. aliquots. The bottles were stoppered with white latex rubber stoppers, size 0, and incubated at  $37^{\circ}C$  until complete monolayers had formed.

Toronto A26/61 virus of known titre ( $1 \times 10^{5.5}$   $TCID_{50}/ml.$ ) was serially diluted (ten-fold) from  $1 \times 10^{-1}$  to  $1 \times 10^{-5}$  in Hanks' balanced salt solution.

Prior to inoculation of the complete monolayers of PDK cells with the different dilutions of Toronto A26/61 virus the medium was discarded and the cell sheets were washed three times with Hanks' balanced salt solution. 0.3 ml. of the different virus dilutions, ranging from  $1 \times 10^{-1}$  to  $1 \times 10^{-5}$ , was added to duplicate bottles. Two control bottles were inoculated with 0.3 ml. of Hanks' balanced salt solution. The virus was allowed to adsorb to the tissue culture monolayers for two hours at  $25^{\circ}C$  with





agitation every fifteen minutes.

The following nutritive overlay media were examined using the Toronto A26/61 virus-PDK cell host system:

- 1) ELY with 2% calf serum added
- 2) ELY " 10% " " "
- 3) HLA " 2% " " "
- 4) EHB " 2% " " "
- 5) 199 " 2% " " "
- 6) EEB " 2% " " "
- 7) EPP " 2% " " "

The media, just prior to mixing with agar, were placed in a 37°C incubator. Autoclaved, 3% special Noble agar was maintained at 43°C to prevent solidification.

After the period allowed for viral adsorption, 10.0 ml. aliquots of equal volumes of nutritive medium and 3% Noble agar (pH = 7.0) were added to the Toronto A26/61 virus-infected PDK cell sheets. The bottles were stoppered and were placed flat side down on a level table (insuring a uniform thickness of agar) until solidification of the agar overlay was complete. The cultures were incubated at 37°C for not less than eleven days, or until it became obvious that the cell sheet was no longer viable. At the end of the incubation period 1.0 ml. of a 0.02% solution of neutral red was applied to each overlay and the overlays were incubated for a further six hours at 37°C in a standard walk-in



incubator. The neutral red diffused through the agar and stained the viable cells. Areas of cells destroyed by the virus appeared as unstained circular plaques. These plaques were counted and were correlated with the dilution to give an estimate of the number of plaque-forming units contained within the original suspension of Toronto A26/61 virus.

Plaque assay results obtained using primary dog kidney cells were unsatisfactory because the cell monolayers degenerated before the appearance of visible plaques. The overlay media became acidic approximately four days after application of the agar overlay. These acidic conditions were believed responsible for the degeneration of the monolayers. Therefore, primary dog kidney cells were not used for subsequent virus assay.

#### Plaque Assay Using DKL Cells

DKL cells were tested for plaque production by Toronto A26/61 virus. These cells were propagated routinely in EEB containing a vitamin supplement (Eagle, 1955) 50 i.u./ml. of penicillin and 50 ug./ml. of streptomycin plus 10% calf serum. Variations in the concentrations and treatments of different additives incorporated into the above nutritive medium were examined for their ability to enhance plaque formation. These were:

- 1) Various concentrations of calf serum, both normal and heat inactivated (held at 56°C for one hour) were



added to the nutritive fraction of the overlay medium. 2%, 5%, and 10% concentrations of calf serum were added to the respective agar overlays.

- 2) Phenol red, which is routinely added to the EEB nutritive medium in a concentration of 2 mg./ml., was on one occasion excluded from the nutritive agar overlay medium to assess the effect, if any, on cell sheet longevity.
- 3) The vital dye (neutral red) was added to the special Noble agar in a 0.01% concentration prior to the addition of the agar overlay to the infected cell sheet. Also, in certain instances, the vital dye was added to the agar overlay at the completion of the incubation period in a 0.02% solution, 1.0 ml./tissue culture vessel.
- 4) Eagle's vitamin supplement (see Appendix #3) was added to the final nutritive agar overlay medium so that the concentration was normal with respect to the formulation reported by Eagle (1955). (This supplement contains eight vitamins in various concentrations.) Another experiment was conducted wherein the concentration of the vitamins was double the concentration reported by Eagle (1955).
- 5) The effect of Eagle's essential amino acid supplement (see Appendix #4) on plaque formation was examined at





normal and twice normal concentrations as described in his formulation (Eagle, 1955).

- 6) Glutamine was examined for its effect on plaque formation. 292 mg./litre and 584 mg./litre in the final nutritive agar overlay medium were the concentrations used.

The additives were added to the nutritive agar overlay medium aseptically after balanced salt solutions and agar had been autoclaved and cooled (37°C and 43°C respectively). Different concentrations of calf serum had little noticeable effect on plaque size, but infected monolayers containing a 10% concentration of calf serum were less acidic and the cell sheets presented a healthier appearance than those cell sheets where lesser amounts of calf serum had been added. Calf serum, whether heated to 56°C for 30 minutes or not heated, produced no observable effect with regard to plaque size or number.

No detectable alteration in the cell monolayer was observed when phenol red was omitted from the nutritive agar medium, as compared with experiments where phenol red was incorporated into the overlay material.

The infected monolayer did not remain viable when the vital dye (0.01% final concentration) was added to the agar overlay material at the time of application of the overlay to the infected monolayers. Superior defi-



nition of plaques was observed when 1.0 ml. of a 0.02% solution of neutral red was added to the titration system after the incubation period. The plaques were more clearly observable three to six hours after application of the neutral red dye than if the system was incubated overnight at 37°C after application of the dye.

The results of the effects on plaque enhancement, by the addition of different concentrations of (1) Eagle's vitamin supplement, (2) Eagle's amino acid supplement, and (3) glutamine, are summarized in Figure #1. The concentrations of additives are indicated within each bar on the graph, as follows: V = vitamins; G = glutamine; Aã = amino acids.

The criterion for the enhancement of plaque formation is based on the diameters of the observable plaques. It may be seen in Figure #1 that the enhancement of plaque formation does not necessarily result in a higher titre of virus. Double the normal concentration of amino acids produced the largest plaques.

#### Effects of Varying Concentrations of Two Chemical Additives on the Enhancement of Plaque Formation

Two chemical additives, (1) diethylaminoethyl-dextran (DEAE-dextran), and, (2) protamine sulfate, known for their ability to enhance plaque size of several arboviruses in human tissue culture systems, (Miles and Austin,



1963), as well as encephalomyocarditis (EMC) and L-cells (Liebhaber and Takemoto, 1963), mengo virus and L-cells (Colter et al, 1964), were tested with the Toronto A26/61 virus and DKL cell system.

These additives were prepared as follows:

DEAE-dextran was dissolved in ion-free water to give a 1.0% solution and sterilized by filtering through a millipore filter, pore size 0.45  $\mu$ . The solution was added to the 3% special Noble agar solution to give the following concentrations: 0  $\mu$ g./ml.; 50  $\mu$ g./ml.; 100  $\mu$ g./ml.; 150  $\mu$ g./ml.; 200  $\mu$ g./ml.

The various overlay media each containing a different concentration of DEAE-dextran, were then added to EEB containing 50 i.u./ml. penicillin, 50  $\mu$ g./ml. streptomycin, 10% calf serum with double the normal concentration of Eagle's amino acid supplement. DKL cells infected with Toronto A26/61 virus were overlayed with the above preparations and were incubated at 37°C for ten days.

The results of these experiments are summarized in Figure #2. The optimum concentrations of DEAE-dextran for the enhancement of plaque size were found to be between 100 to 150  $\mu$ g./ml.

In a similar manner, protamine sulfate was prepared in a 1.0% solution in ion-free water and was





filter sterilized using a millipore filter. The protamine sulfate was added to the 3% special Noble agar solution so that the final concentrations of the additive in final overlay medium were 0 ug./ml; 50 ug./ml.; 100 ug./ml.; 150 ug./ml.; 200 ug./ml. The results of the effects of various concentrations of protamine sulfate on plaque size are summarized in Figure #3.

The optimum concentration of protamine sulfate for the enhancement of plaque size was 75 ug./ml. The experiment wherein 200 ug./ml. of protamine sulfate was used failed to give useful results. Plaques were ill-defined and could not be counted.

The results of the comparison of the PFU method of virus titration with the TCID<sub>50</sub> method of virus titration are summarized in Figure #4. The comparison was made from a series of twenty separate experiments where both methods of titration were used..

Conditions under which experiments #1 to #5 were conducted were not as carefully controlled with respect to nutritive agar overlay material as were the conditions under which experiments #6 to #20 were conducted. This control is reflected in the variation between PFU titres when compared with TCID<sub>50</sub> titres. PFU titres approximate TCID<sub>50</sub> titres to a lesser degree in the first five experiments (differences vary from 1 to 4 logs). In the last



fifteen experiments, PFU titrations and TCID<sub>50</sub> titrations are within the same log.

#### THE EFFECT OF CELL CONCENTRATION ON VIRUS PRODUCTION

Green and Pina (1963a) used suspension cultures of KB cells to propagate human adenoviruses types 2 and 4 with satisfactory results. It was decided to determine whether or not suspension cultures of DKL cells were satisfactory for the propagation of Toronto A26/61 virus.

A suspension (100 ml.) of DKL cells at a density of  $1 \times 10^6$  cells/ml. was inoculated with Toronto A26/61 virus exhibiting a titre of  $1 \times 10^{6.9}$  TCID<sub>50</sub>/ml. so that the concentration of inoculum was 1 TCID<sub>50</sub>/cell approximately. The cell-virus mixture was incubated at 37°C for two hours, under constant agitation. Aliquots of the above mixture were diluted to the desired cell concentrations (see Table #6) using EEB medium containing 50 i.u./ml. penicillin, 50 ug./ml. streptomycin, 10% calf serum, Eagle's vitamin supplement and twice the normal concentration of Eagle's amino acid supplement, plus glutamine. The cell concentrations examined are shown in Table #6, page 56.



Table #6

<u>Flask No.</u>	<u>Initial Concentration</u>	<u>Final Concentration</u>	<u>Yield In PFU/0.3 ml. × 10<sup>3</sup></u>
1	250,000 cells/ml.	337,500 cells/ml.	-
2	500,000 cells/ml.	527,500 cells/ml.	18
3	750,000 cells/ml.	787,500 cells/ml.	55
4	1,000,000 cells/ml.	1,012,000 cells/ml.	13

The infected DKL cell cultures (100 ml.) were placed in sterile 250 ml. Erlenmyer flasks containing a sterile bar magnet. The flasks were then placed over a "Magnastir" apparatus and incubated for 48 hours at 37°C.

After the incubation period the flasks were placed in a freezer (-20°C) and subsequently the cells were frozen and thawed three times. The cells were sedimented in an International centrifuge (Model SB, head 259, 25°C) at 2,000 rpm. for fifteen minutes. The cell pellets were then resuspended in 10 ml. of their original nutritive medium and were pipetted up and down vigorously ten times in order to dislodge virus from the disrupted cells. Each of the four 10 ml. suspensions was centrifuged at 2,000 rpm. for ten minutes and the supernatants were recombined with their respective 90 ml. culture supernatants.

The supernatants representing each cell con-





centration were assayed by the PFU method of assay using 75 ug./ml. of protamine sulfate in the special Noble agar.

The yield of virus is represented in Figure #5. Titres are in PFU/0.3 ml.  $\times 10^3$ , cell concentrations appear as shown. An initial cell concentration of 750,000 cells/ml. appeared to produce the best yield of virus, but this yield was still very low as compared with yields obtained from monolayer cultures infected with Toronto A26/61 virus. The yield obtained from the preparation containing 750,000 cells/ml. was  $1.6 \times 10^5$  PFU/ml. or a yield of approximately 2 PFU for every 10 cells as compared with an inoculum concentration of 1 TCID<sub>50</sub>/cell.

Yields for monolayer cultures varied from  $1 \times 10^7$  to  $1 \times 10^8$  TCID<sub>50</sub>/ml., or approximately 4 to 8 TCID<sub>50</sub>/cell using a similar inoculum concentration of 1 TCID<sub>50</sub>/cell.

#### THE EFFECT OF VIRUS CONCENTRATION ON VIRUS PRODUCTION

DKL cells were seeded into three-ounce prescription bottles at a concentration of 250,000 cells/ml. and incubated at 37°C until complete monolayers had formed (two days). The cell sheets on two representative three-ounce bottles were treated with versene to remove the cells for counting with a hemocytometer. The average count was  $9.6 \times 10^6$  cells per bottle.

Toronto A26/61 virus, exhibiting a titre of  $1 \times 10^{7.75}$  TCID<sub>50</sub>/ml. was added to the monolayers in ml. quantities until the following virus-to-cell ratios were obtained:

- 1) 6 TCID<sub>50</sub>/cell



- 2) 10 TCID<sub>50</sub>/cell
- 3) 20 " "
- 4) 25 " "
- 5) 30 " "

After inoculation the infected monolayer cells were incubated for two hours at 25°C with agitation every fifteen minutes. After a two hour adsorption time, additional nutritive medium was administered and the monolayers incubated at 37°C for 48 hours.

Following incubation the monolayers were removed to a -20°C freezer where they were frozen. They were then removed, thawed, and returned to the freezer. This was done three times. The cells and the nutritive medium contained within the three-ounce bottles were pipetted vigorously to further rupture the cells. 0.2 ml. of each virus concentration (mentioned above) was added to 1.8 ml. aliquots of Hanks' balanced salt solution. Serial ten-fold dilutions from  $1 \times 10^{-1}$  to  $1 \times 10^{-10}$  were made. 0.1 ml. of each dilution was inoculated into tube monolayer cultures in triplicate. Observations\* were recorded every two days for a total of 18 days.

A similar experiment was performed using PDK cells with the following virus-to-cell ratios:

- 1) 1 TCID<sub>50</sub>/cell
- 2) 10 " "
- 3) 25 " "

\*Observations were for C.P.E.



- 4) 50 TCID<sub>50</sub>/cell
- 5) 100 " "
- 6) 125 " "
- 7) 150 " "

The results from these studies are summarized in Figures #6 and #7. The yields of virus are given in TCID<sub>50</sub>/ml. It will be noticed that very little difference in yield is evident, regardless of the relative concentrations of virus in an inoculum in either DKL or PDK cells.

#### CONCENTRATION OF TORONTO A26/61 VIRUS

An increase in total infectivity, an increase in total hemagglutinin, or an increase in optical density at 260 mμ., were the criteria used as an indication to the degree of concentration of Toronto A26/61 virus employing the following five methods:

##### 1. Concentration by Differential Centrifugation

Toronto A26/61 virus was propagated on monolayers of DKL cells contained within Roux culture bottles. After complete monolayers of DKL cells had formed, the nutritive medium was discarded and the monolayers were inoculated with a suspension of Toronto A26/61 virus so that the virus content of the inoculum was approximately 25 TCID<sub>50</sub>/cell. The viral suspension was allowed to adsorb to DKL cells for two hours at 25°C with agitation every fifteen minutes. After the adsorption period, fresh nutritive medium was added prior to incubation at 37°C for 48 hours. The infected cultures were detached by freezing







and thawing. The cell debris was sedimented at 2,000 rpm. using the #259 head in the International Model SB centrifuge for fifteen minutes at 25°C and the pellet was frozen and thawed four more times in 20 ml. of nutritive medium and recentrifuged. The two supernatants containing the virus were pooled to give a total volume of 1,750 ml. of virus suspension. The titre of this virus suspension was  $1 \times 10^{7.5}$  TCID<sub>50</sub>/ml. This virus suspension was centrifuged at 10,000 rpm. for ten minutes (to further remove cell debris) using the SS34 rotor in the RC2 Sorvall centrifuge. The titre following this centrifuging was identical to that before centrifugation. 1,500 ml. of the supernatant from the above centrifugation were placed in six 250 ml. polyethylene centrifuge bottles and centrifuged at 19,000 rpm. for two hours using the type 19 rotor in the Beckman L-2 preparative ultracentrifuge (250 ml. were retained as a stock supply of Toronto A26/61 virus, titre  $1 \times 10^{7.5}$  TCID<sub>50</sub>/ml. The pellet containing the virus was resuspended in 15 ml. of 0.04 M. phosphate buffer pH 7.0. The titre of this suspension of Toronto A26/61 virus was determined. A comparison of virus recovery was determined on basis of infectivity (see Table #7, page 61)



Table #7

<u>Centrif.</u>	<u>Supernatant</u>	<u>Centrif. Velocity</u>	<u>Infectivity in TCID<sub>50</sub>/ml</u>	<u>Total Infect. of Supernatant</u>
1	1750 ml.	2000 rpm/15 min	$1 \times 10^{7.5}$ $= 3.2 \times 10^7$	$5.6 \times 10^{10}$
2	1750 ml.	10000 rpm/10 min	$1 \times 10^{7.5}$ $= 3.2 \times 10^7$	$5.6 \times 10^{10}$
3	1500 ml.	19000 rpm/120 min	$1 \times 10^{5.25}$ $= 1.8 \times 10^5$	$2.7 \times 10^8$
4	15 ml. viral pellet from (3)	--	$1 \times 10^{7.6}$ $= 4 \times 10^7$	$6.0 \times 10^8$

The % recovery of infectivity contained in pellet from centrifugation # (3) when it was resuspended in 15 ml. of 0.04 M. phosphate buffer pH 7.0 was calculated in the following manner:

- original total infectivity of the 1500 ml. was

$$3.2 \times 10^7 \text{ TCID}_{50}/\text{ml.} \times 1500 = 4.8 \times 10^{10} \text{ TCID}_{50}$$

- final total infectivity of the 15 ml. resuspended pellet was

$$4.0 \times 10^7 \text{ TCID}_{50}/\text{ml.} \times 15 = 6 \times 10^8 \text{ TCID}_{50}$$

Therefore, % recovery was

$$100 \times \frac{6.0 \times 10^8}{4.8 \times 10^{10}} = \frac{6.0}{480} \times 100 = 1.25\%$$

The total infectivity of the 1500 ml. supernatant after centrifugation at 19000 rpm/120 min. was  $2.7 \times 10^8 \text{ TCID}_{50}$  as compared with the infectivity prior to centrifugation of  $4.8 \times 10^{10} \text{ TCID}_{50}$ . Therefore, total infectivity was reduced by almost three logs.



The % difference between total infectivity at centrifugation # (3) and centrifugation # (4) was

$$100 \times \frac{4.8 \times 10^{10} - 2.7 \times 10^8}{4.8 \times 10^{10}} = \frac{477.3}{480} \times 100 = 99.4\%$$

Most of the infectivity was lost during centrifugation.

## 2. Concentration by Column Chromatography Using DEAE-Cellulose

DEAE-cellulose was mixed with 0.04 M. phosphate buffer, pH 7.0, to give approximately a 20% suspension. The slurry was added to a column, 1.0 cm. in diameter, to a height of 10.0 cm. The column was washed with 10.0 ml. volumes of 0.04 M. phosphate buffer several times.

1.0 ml. of Toronto A26/61 virus containing  $1 \times 10^{7.6}$  TCID<sub>50</sub> in 0.04 M. phosphate buffer was added to the column. The virus suspension was followed with three successive 10 ml. aliquots of 0.04 M. phosphate buffer. The virus was subsequently eluted stepwise from the column with 5.0 ml. aliquots of 0.04 M. phosphate buffered (pH 7.0) sodium chloride solutions with the following concentrations: 0.05 M; 0.10 M; 0.15 M; 0.20 M; 0.25 M; 0.30 M; 0.35 M; 0.40 M; 0.45 M; 0.50 M; 0.60 M; 0.70 M; 0.80 M; 0.90 M; 1.0 M.

The fractions eluted from the column were collected in 13 x 100 mm. test tubes and scanned spectrophotometrically (between 200 mu. and 340 mu.) using the Bausch and Lomb #505 recording spectrophotometer. Fractions exhibiting absorbance at





260 mu. were titrated for infectivity and hemagglutinin. The results of these examinations are summarized in Figure #8.

The major portion of the infectivity resided in fractions with sodium chloride concentrations of 0.2 M. to 0.4 M. as determined by conductivity measurements. The hemagglutinin was in a range varying between 0.2 M. to 0.6 M. sodium chloride concentration. The peak displayed at a sodium chloride concentration of 0.7 M. was probably nucleoprotein from host cell sources. Infectivity and hemagglutinin were not found in this fraction.

The per cent recovery of infectivity using DEAE-cellulose column chromatography as a method for the concentration of Toronto A26/61 virus was:

- total infectivity added to the column  $1 \times 10^{7.6} = 4 \times 10^7 \text{ TCID}_{50}$
- total infectivity recovered from the column  $13.6 \times 10^6 \text{ TCID}_{50}$

$$\frac{13.6 \times 10^6}{40.0 \times 10^6} \times 100 = 34\%$$

The per cent recovery of hemagglutinin was

- total amount of hemagglutinin added to the column 16,384 hemagglutination units
- total amount of hemagglutinin recovered from the column 2,720

$$\frac{2720}{16384} \times 100 = 16.7\%$$



### 3. Concentration by Filtration on Sephadex G200 Gel

4.0 grams of Sephadex (G200) were added to 200 ml. of 0.04 M. phosphate buffer (pH 7.0) and the suspension was allowed to equilibrate for three hours. The Sephadex slurry was then added to a 1.0 cm. column to a height of 5.0 cm. The column was washed through several times with 0.04 M. phosphate buffer. 1.0 ml. of Toronto A26/61 virus ( $1 \times 10^{7.6}$  TCID<sub>50</sub>/ml.) was added to the column. 5.0 ml. aliquots of 0.04 M. phosphate buffer (pH 7.0) were added to the column. Eight separate fractions were collected in 13 x 100 mm. test tubes. Each fraction was subjected to a spectrophotometric analysis (between 200 mu. and 340 mu.) and titrated for infectivity. The results obtained from Sephadex G200 gel filtration of Toronto A26/61 virus are summarized in Figure #9. Fraction #1 contained 69% of the original infectivity; fractions #2, #3, #4, and #5 had essentially no infectivity. Fractions #6, #7, and #8 were collected two, four, and six days after fractions #1 to #5. Titrations of these samples were attempted, but due to bacterial contamination, accurate titres could not be determined. Titres ranged from  $10^4$  to  $10^5$  TCID<sub>50</sub>/ml.

### 4. Concentration by Tris Genetron Extraction

Green and Pina (1963a) effectively used genetron (tri-fluoro trichloro ethane)(CCl<sub>2</sub>F-CClF<sub>2</sub>) in conjunction with Tris-HCl buffer, pH 8.1, to extract and initially purify human adenovirus types 2 and 4 from KB culture cells.



It was decided to try this procedure for the initial concentration of Toronto A26/61 virus from DKL cells. Four Roux bottles were inoculated with Toronto A26/61 virus at a concentration of approximately  $20 \text{ TCID}_{50}/\text{cell}$ . The bottles were incubated for 48 hours at  $37^{\circ}\text{C}$ . Cell sheets were removed from the bottles (with a rubber policeman) and centrifuged at 2,000 rpm. in the International Model SB centrifuge (using #259 head) at  $25^{\circ}\text{C}$  for fifteen minutes. The pellets were resuspended in 10 ml., 0.01 M. Tris HCl buffer, pH 8.1, and stored at  $-20^{\circ}\text{C}$  until required. The infected cell pellet was frozen and thawed three times. An additional 35 ml. of Tris-HCl buffer was added making a total of 45 ml. The suspension was pipetted up and down vigorously ten times and was centrifuged in the International Model SB centrifuge in the #259 head at  $25^{\circ}\text{C}$  at 2,000 rpm. for fifteen minutes. The supernatant was homogenized with 45 ml. of genetron. The mixture was homogenized in the Waring blender at 10,000 rpm. for five minutes. The container was immersed in an ice bath during the homogenization. The aqueous and organic layers were separated by centrifugation at 2,000 rpm. for five minutes in the International centrifuge. The aqueous upper layer was removed with a pipette, homogenized again with 30 ml. of fresh genetron and the layers were again separated by centrifugation. The genetron layers from the two extractions were combined, homogenized with 20 ml. of Tris buffer (0.01 M., pH 8.1) and centrifuged as above. The







aqueous layer was added to the combined aqueous layers of the first two centrifugations. This suspension was subjected to absorbance (on the Bausch and Lomb #505 recording spectrophotometer) and infectivity studies.

Figure #10 shows the absorbance studies on the Tris-genetron extract of Toronto A26/61 virus-infected DKL cells. The original titre of the extract prior to genetron treatment was  $1 \times 10^{7.75}$  TCID<sub>50</sub>/ml. The titre after extraction was zero. This could mean that the absorbance was due to unorganized nucleoprotein rather than virus (see Figure #10, line #1). The fraction assayed was subsequently subjected to deoxyribonuclease and ribonuclease, centrifuged and resuspended and the optical density was observed to be almost zero (see Figure #10, line #2). These results would tend to verify the fact that the isolate was a nucleoprotein.

#### 5. Concentration by Buoyant-Density Gradient Centrifugation

Virus-infected cells were prepared as previously described in this section under "Concentration by Differential Centrifugation."

A cesium chloride solution ( $\rho = 1.400$ ) was prepared in 0.04 M. phosphate buffer (pH. 7.0). 5.0 ml. of this solution were placed in a 5.0 ml. cellulose nitrate centrifuge tube. The partially concentrated Toronto A26/61 virus pellet was added to the buffered cesium chloride solution. The total infectivity of the virus suspension was  $5 \times 10^7$  TCID<sub>50</sub>. The tube was centrifuged in the type 39 rotor of the Beckman L-2 preparative ultracentrifuge at 35,000 rpm. for 24 hrs. at 6°C. A well defined band



was observed approximately 10 mm. from the bottom of the tube. A hole was punched in the bottom of the tube with a 20 gauge hypodermic needle and successive five-drop fractions collected. The fractions were analyzed for infectivity, hemagglutinin, absorbance (at 260 mu.) and buoyant-density. The results of the above studies are presented in Figures #11 and #12. The infectivity was confined to a narrow, defined band and extended over four fractions. The total infectivity in these four fractions was  $2.6 \times 10^7$  TCID<sub>50</sub>. Therefore, the per cent recovery of Toronto A26/61 virus infectivity using this method of concentration was:

$$\frac{2.6 \times 10^7}{5 \times 10^7} \times 100 = 51\%$$

The absorbance curve displayed by a Toronto A26/61 virus suspension after concentration by the above method and subsequent treatment with DNase to remove any adsorbed DNA appears in Figure #14.

A further description of results on buoyant-density studies follows.

#### BUOYANT-DENSITY STUDIES

As previously mentioned, viruses centrifuged to equilibrium in a cesium chloride gradient will band at a density identical with their own in the gradient. It was decided to compare Toronto A26/61 virus with the other canine adenovirus (infectious canine hepatitis, ICH) with regard to buoyant-density. Crawford's (1960) procedures were followed using



cesium chloride instead of rubidium chloride.

#### Buoyant-Density Comparison Between Toronto A26/61 and ICH Viruses

Infectious canine hepatitis and Toronto A26/61 viruses were propagated in DKL cells in Roux bottles. The viruses were harvested and concentrated as previously described under "Concentration by Differential Centrifugation" and "Concentration by Buoyant-Density Centrifugation."

The fractions collected after concentration by buoyant-density centrifugation were weighed in a 100 lambda pipette on the Mettler Type H6T balance. An equal volume of de-ionized water was also weighed and the density for each fraction was computed. These densities were correlated with hemagglutinin and infectivity titrations.

The results for concentration of Toronto A26/61 virus by buoyant-density centrifugation were combined with the results of the study of the buoyant-densities of Toronto A26/61 and ICH viruses.

Plate #6 shows a photograph of the tubes after centrifugation. Because of the presence of more virus in the Toronto A26/61 virus tube a more defined set of density bands is visible as compared with the ICH virus tube. The dark lower band was correlated with infectivity and was therefore believed to be the viral band. The upper band was believed to be aberrant viral particles in some cases lacking nucleic acid. Hemagglutinin was still demonstrable in this band.







The bands in the ICH virus tube closely paralleled the bands in the Toronto A26/61 virus tube and cytopathogenicity correlated with a buoyant-density of 1.333 (see Figure #13). Because of the relatively small amount of virus present in the ICH virus tube, low optical density readings were obtained.

A second experiment with Toronto A26/61 virus showed that cytopathogenicity and optical density both appeared at a buoyant-density of 1.334.

Peak absorbance at 260 m $\mu$ ., infectivity, and hemagglutinin appeared between Fraction #3 to #6. Peak absorbance at 260 m $\mu$ . corresponded with a buoyant-density of 1.330 in Figure #11 and 1.334 in Figure #12.

Figure #13 shows the results of assay of the ICH virus buoyant-density study.

Cytopathogenicity corresponded with tube #6 which exhibited a buoyant-density of 1.333.

Toronto A26/61 and ICH viruses have similar buoyant-densities varying from 1.330 to 1.334, which is within experimental error for equal buoyant-densities.

#### The Relationship Between the Buoyant-Density of Deoxyribonucleic Acid and the Mole Fraction % (G + C)

The results of the density studies on salmon sperm DNA when subjected to density-gradient centrifugation to equilibrium appear in Figure #21. The calculation of buoyant-



density from the mole fraction % (G + C) (Schildkraut et al, 1962) is as follows:

$$\text{buoyant-density} = 0.098 (G + C) + 1.660 \text{ gm./cu. cm.}$$

$$(G + C) = \text{mole fraction \% (G + C)}$$

Salmon sperm DNA exhibits purine and pyrimidine base ratios of 20.8; 20.4; 29.7; 29.1, for guanine, cytosine, adenine, and thymine respectively (mole fraction % (G + C) = 41.2%) (Chargaff and Davidson, 1955). The buoyant-density can be calculated with this information as follows:

$$\text{buoyant-density} = (0.098) (41.2) + 1.660 \text{ gm./cu. cm.}$$

$$= 1.701 \text{ gm./cu. cm.}$$

The experimental value for the buoyant-density of salmon sperm DNA was 1.704 (Figure #21). This density is that of fraction #18 which was the first fraction where DNA appeared.

An inspection of Figure #16 reveals a buoyant-density of 1.701 for DKL-cell DNA. The DNA was concentrated in fraction #18. A buoyant-density of 1.701 corresponds to a mole fraction % (G + C) content of 42%. The % (G + C) for DKL-cell DNA, as determined by base ratio analysis, was also 42% (see Table #9).

Figure #17 shows the buoyant-densities of DNA extracted from Toronto A26/61 virus-infected DKL cells. Two peaks absorbing at 260 mu. were obtained -- one peak in fraction #20 and a second peak in fraction #22.



These fractions had buoyant-densities of 1.718 for the first peak and 1.701 for the second peak and corresponded to a % (G + C) content of 59% and 42% respectively (see Figure #16 and Table #9). The first peak probably exhibits the buoyant-density of Toronto A26/61 virus DNA. Since base analysis of the Toronto A26/61 virus DNA would confirm or refute this value, base analysis of the viral DNA was undertaken.

### CHEMICAL STUDIES

Bendich (1957) showed that the liberation of bases from nucleic acids depends on the cleavage of acid-labile glycosidic bonds. Hydrolysis by means of formic or perchloric acid is usually the method of choice for the cleavage of these bonds.

Hydrolysates obtained are applied to filter paper strips and the bases contained therein are separated by an isopropanol-HCl solvent system.

The concentrations of purines and pyrimidines may be determined from the absorption maxima extinctions (Bendich, 1957). The relationship between molar concentration and molecular extinction coefficient ( $\epsilon$ ), and extinction (E) or optical density (OD) is:

$$\text{Molarity} = \frac{E}{\epsilon} = \frac{OD}{\epsilon}$$

Hence, the ratios of guanine, cytosine, adenine, thymine, may be determined in molar percentages. An example of such a calculation appears in Table #8.





Table #8

## CALCULATION OF MOLAR RATIO OF BASES IN DKL-DNA

Bases	A		B		C		(A)-(B)-(C) Corrected O.D. Bases	$\epsilon^* \times 10^{-3}$	(A)-(B)-(C) $\epsilon$	Molar Ratio (%)
	O.D. Bases	0.D. Blank	0.D. Water Blank	0.D. Filter Paper	0.D. Blank	0.D. Filter Paper				
Guanine	0.580		0.000		0.200		0.380	11.1	0.0302	$\frac{0.0302}{0.1542} \times 100 = 19.6$
Cytosine	0.670		0.000		0.170		0.500	10.0	0.0450	$\frac{0.0450}{0.1542} \times 100 = 22.0$
Adenine	0.440		0.000		0.100		0.340	12.6	0.0340	$\frac{0.0340}{0.1542} \times 100 = 29.2$
Thymine	0.520		0.000		0.160		0.360	7.95	0.0450	$\frac{0.0450}{0.1542} \times 100 = 29.2$
									<hr/>	
									0.1542	

Guanine = 19.6%

Cytosine = 22.0%

Adenine = 29.2%

Thymine = 29.2%

\*Bendich (1963)



Absorbance curves of guanine, adenine, cytosine, uracil, and thymine are presented in Figure #22. An absorbance curve of salmon sperm DNA (50 ug./ml.) appears in Figure #23. The  $O.D. 230/O.D. 260$  ratio = 0.41 and the  $O.D. 260/O.D. 280$  ratio = 1.75. An absorbance curve of hydrolysed salmon sperm DNA appears in Figure #24. The mole fraction ratios of bases are as follows: 21, 22, 29, 28, for guanine, cytosine, adenine, thymine, respectively.

Figure #15 shows the absorbance characteristics of DKL-DNA after extraction and purification and subsequent treatment with RNase. The  $O.D. 230/O.D. 260$  ratio = 0.47 and the  $O.D. 260/O.D. 280$  ratio = 1.79.

The absorbance characteristics of hydrolysed DKL-cell DNA appear in Figures #18 and #19. The average mole fraction ratios of bases are 20, 22, 29, 29 for guanine, cytosine, adenine, thymine, respectively.

The absorbance characteristics of hydrolysed Toronto A26/61 virus appear in Figure #20. The mole fraction ratios of bases are: 27, 29, 22, 22, for guanine, cytosine, adenine, thymine, respectively.

The results of a comparison of the chromatograms of standard bases, hydrolysed and chromatographed salmon sperm DNA, Toronto A26/61 virus DNA and DKL-cell DNA are presented on Plate #7.

A summary of the preceeding results appears in



Table #9 together with base ratios taken from other sources as a basis for comparison.

Table #9

Species	Mole Fract. G + C		Mole Fraction Purine & Pyrimidine Bases in DNA				Buoyant- Density
	<u>/Chem</u>	*BDG	Guanine	Cytosine	Adenine	Thymine	
A26/61 +	56	59	27	29	22	22	1.718
A2 ++	56	58 +++	27	29	22	21	1.716 +++
A4 ++	57	59 +++	27	30	22	20	1.718 +++
A12 +++	-	48	-	-	-	-	1.708
A18 +++	-	49	-	-	-	-	1.709
Polyoma +++	-	48	-	-	-	-	1.709
Papilloma +++	-	49	-	-	-	-	1.711
DKL cells +	42	42	20	22	29	29	1.701
Beef Kid. ++++	44	-	23	21	28	28	-
KB Cells ++	42	-	21	21	28	30	-
Sal.sperm +	41	44	21	20	30	29	1.703
Sal.sperm +++++	41	-	21	20	30	29	-

/ Chemical Analysis

\* BDG - buoyant-density gradient

+ Thesis data

++ Green M., Pina M., (1963a) *Virology* 20: 199

+++ Green M., Pina M., (1963b) *Proc. Nat. Acad. Sci.* 50: 44

++++ Daly M.M., Alfrey U.G., Mirsky A.E. (1950)

*J. Gen. Physiol* 33: 497

+++++ Chargaff E., Lipshitz R., Green C., Hodes M.E. (1951)

*J. Biol. Chem.* 192: 223

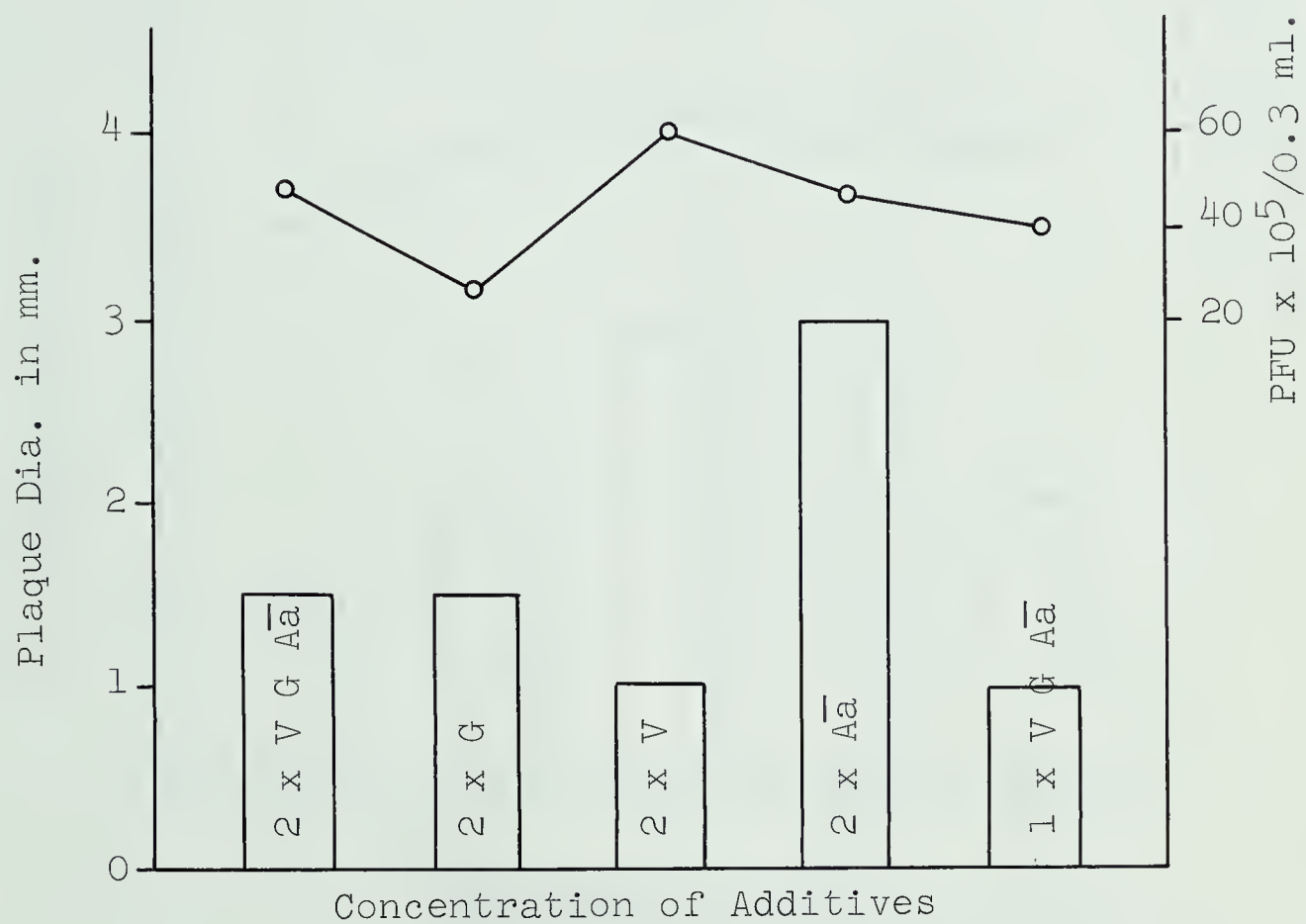
ratios shown are to the nearest whole number





FIGURE #1

THE EFFECT OF VARYING VITAMINS, GLUTAMINE, &  
AMINO ACID CONTENT ON PLAQUE SIZE AND NUMBER  
USING TORONTO A26/61 VIRUS-INFECTED DKL CELLS



V = vitamins

G = glutamine

Aa = amino acids

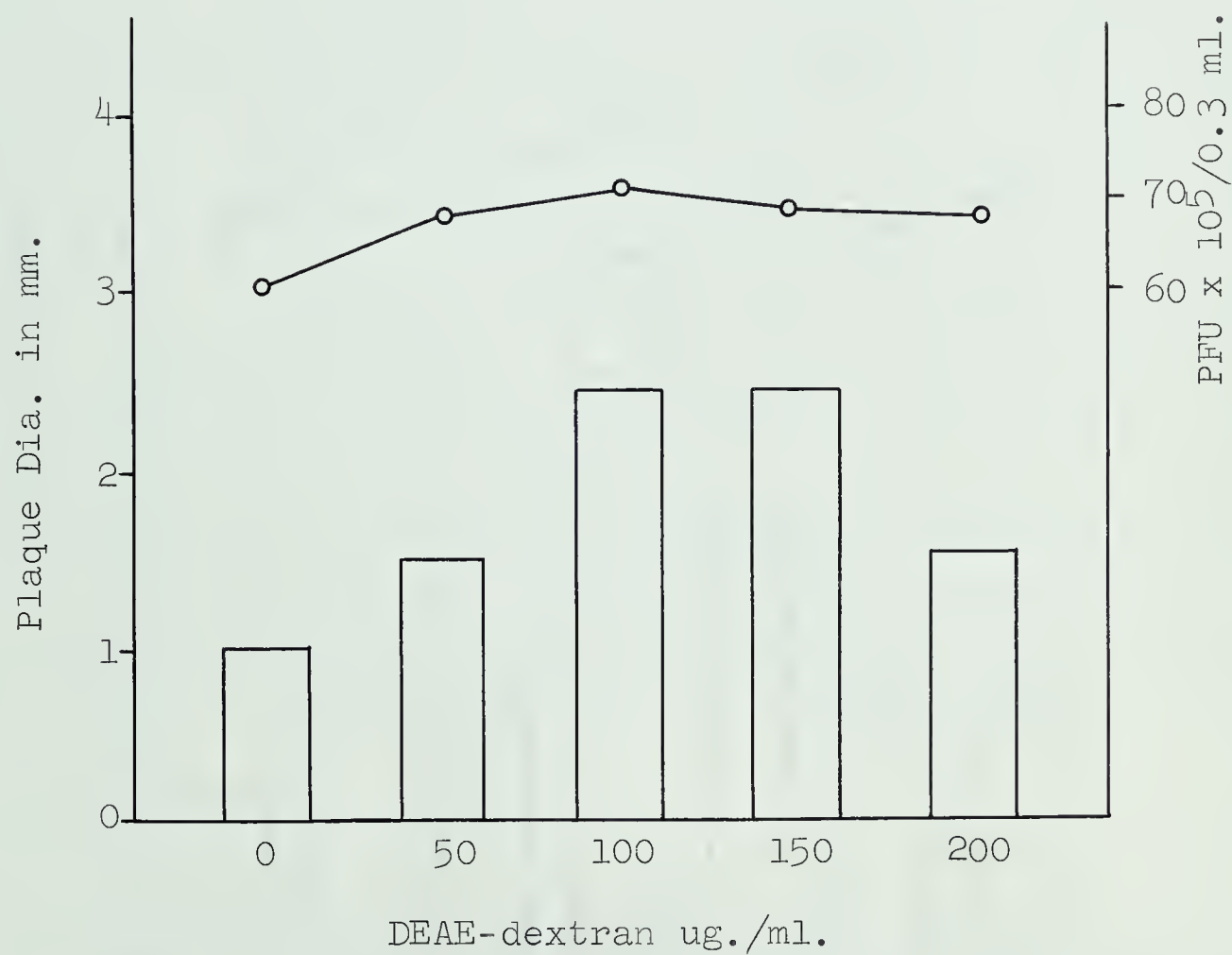
□ = plaque diameter

○—○ = PFU/0.3 ml.



FIGURE #2

The Effect of DEAE-dextran Content On  
Plaque Size and Number Using Toronto A26/61  
Virus-Infected DKL Cells



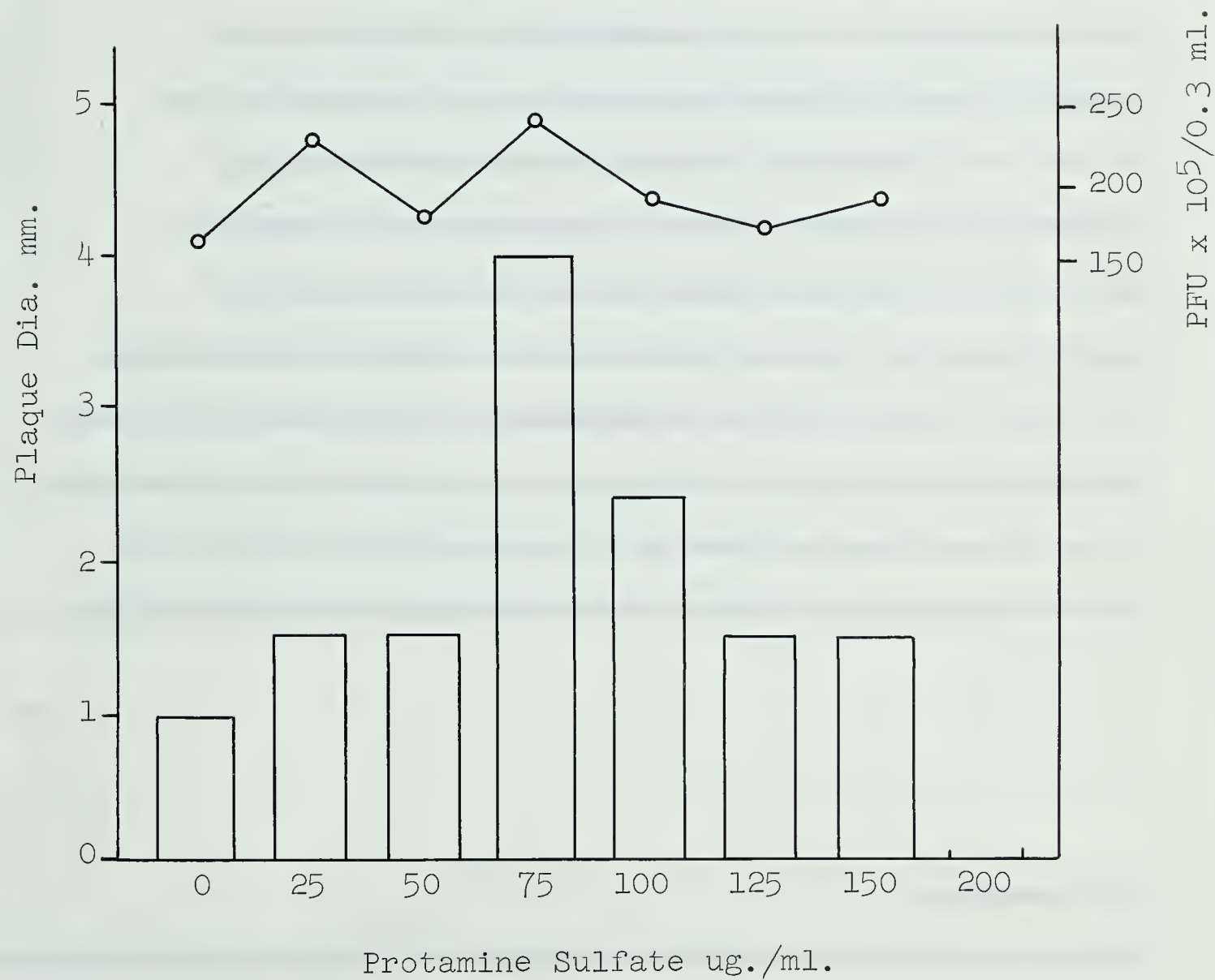
□ = PFU/0.3 ml.

○—○ = plaque diameter



FIGURE #3

The Effect of Protamine Sulfate Content On  
Plaque Size and Number Using Toronto A26/61  
Virus-Infected DKL Cells



□ = PFU/0.3 ml.  
○—○ = plaque diameter





FIGURE #4

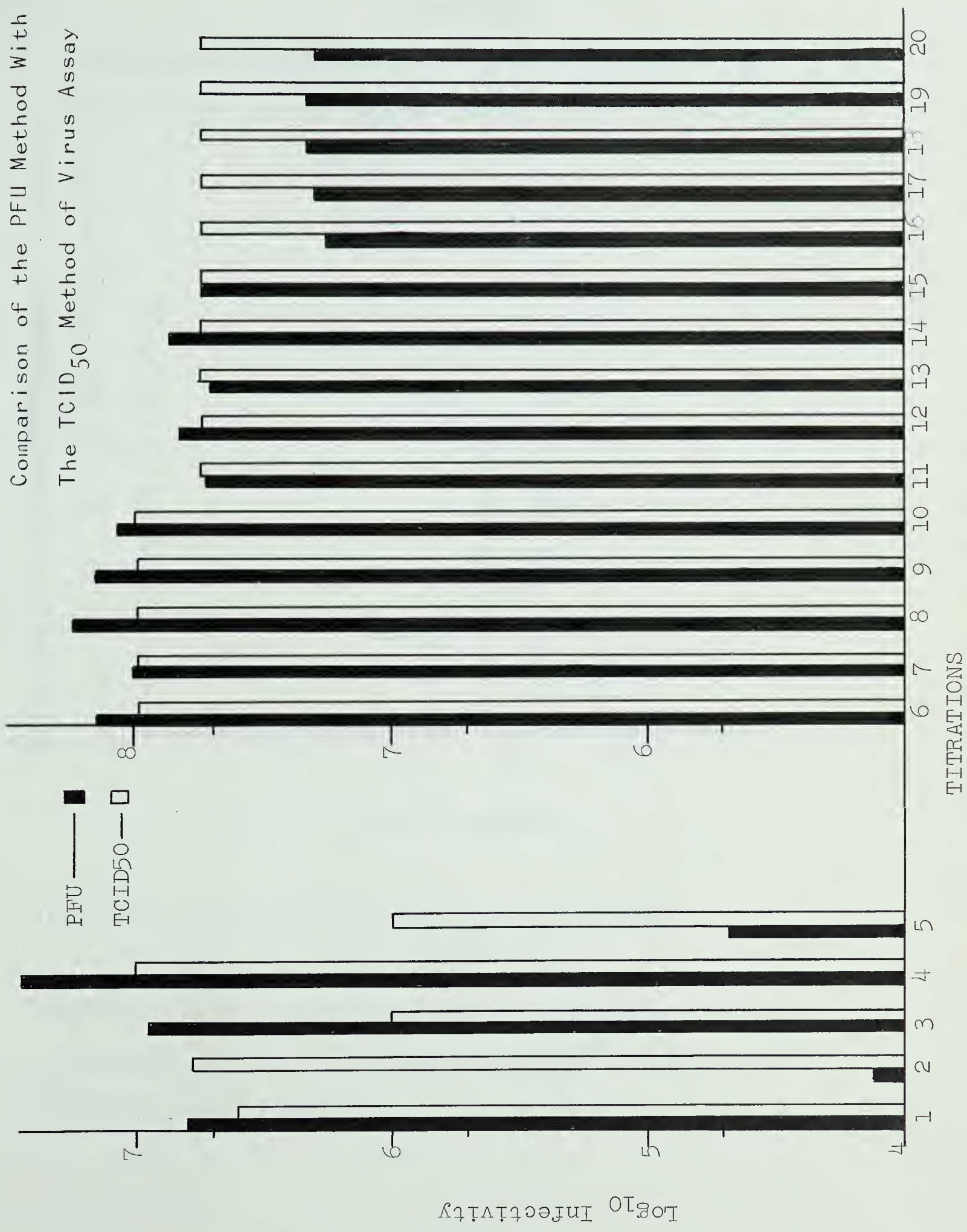
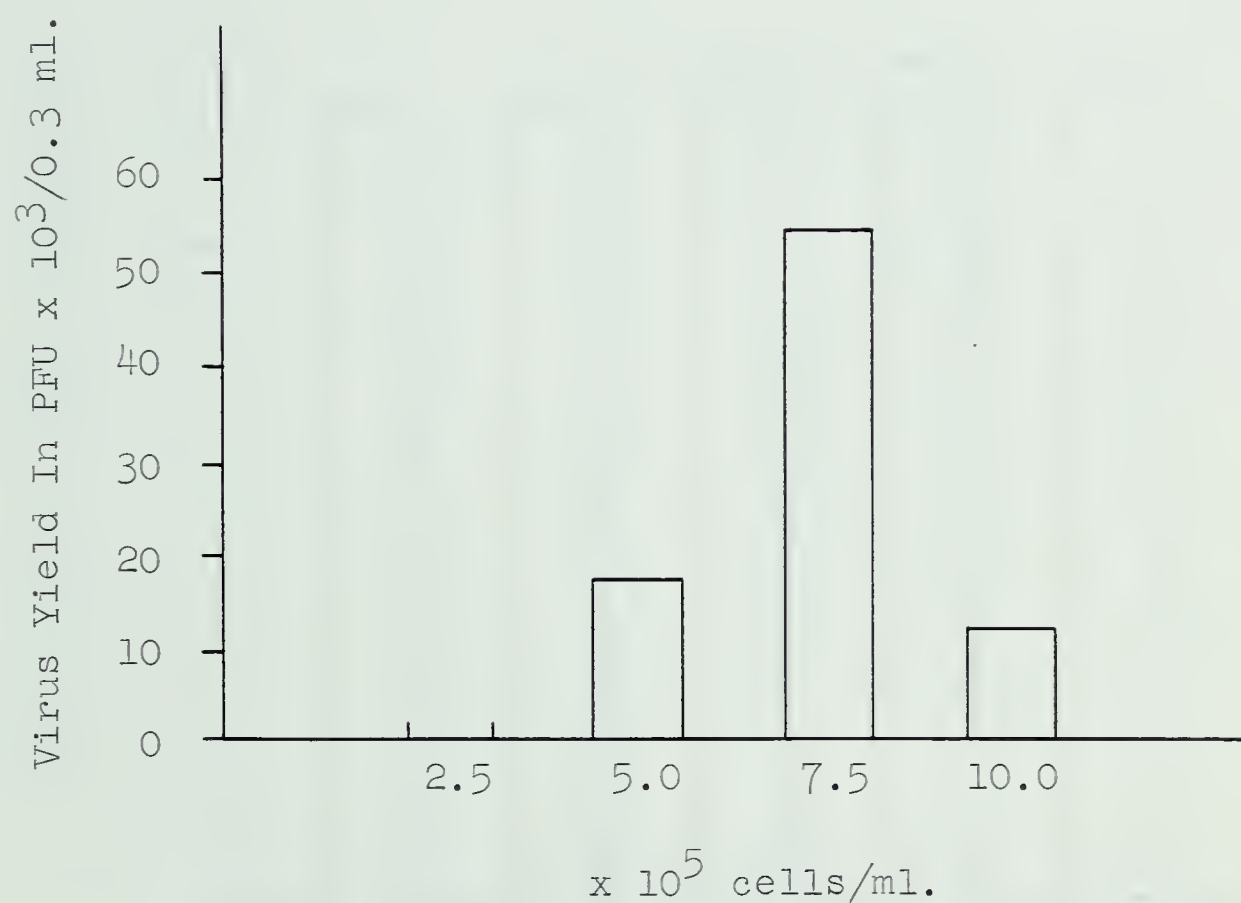




FIGURE #5

The Effect of Cell Concentration on Toronto A26/61  
Virus Production in Suspension Cultures of DKL Cells



The concentration of virus in the inoculum was  
 $1 \times 10^{6.9}$  TCID<sub>50</sub>/ml., or, 1 TCID<sub>50</sub>/cell.

The best yield was obtained from a cell concentration  
of 750,000 cells/ml. which was  $1.6 \times 10^5$  PFU/ml., or  
2 PFU for every 10 cells.



FIGURE #6

Effect of Concentration of Inoculum on Production  
of Toronto A26/61 Virus on Monolayers of DKL Cells

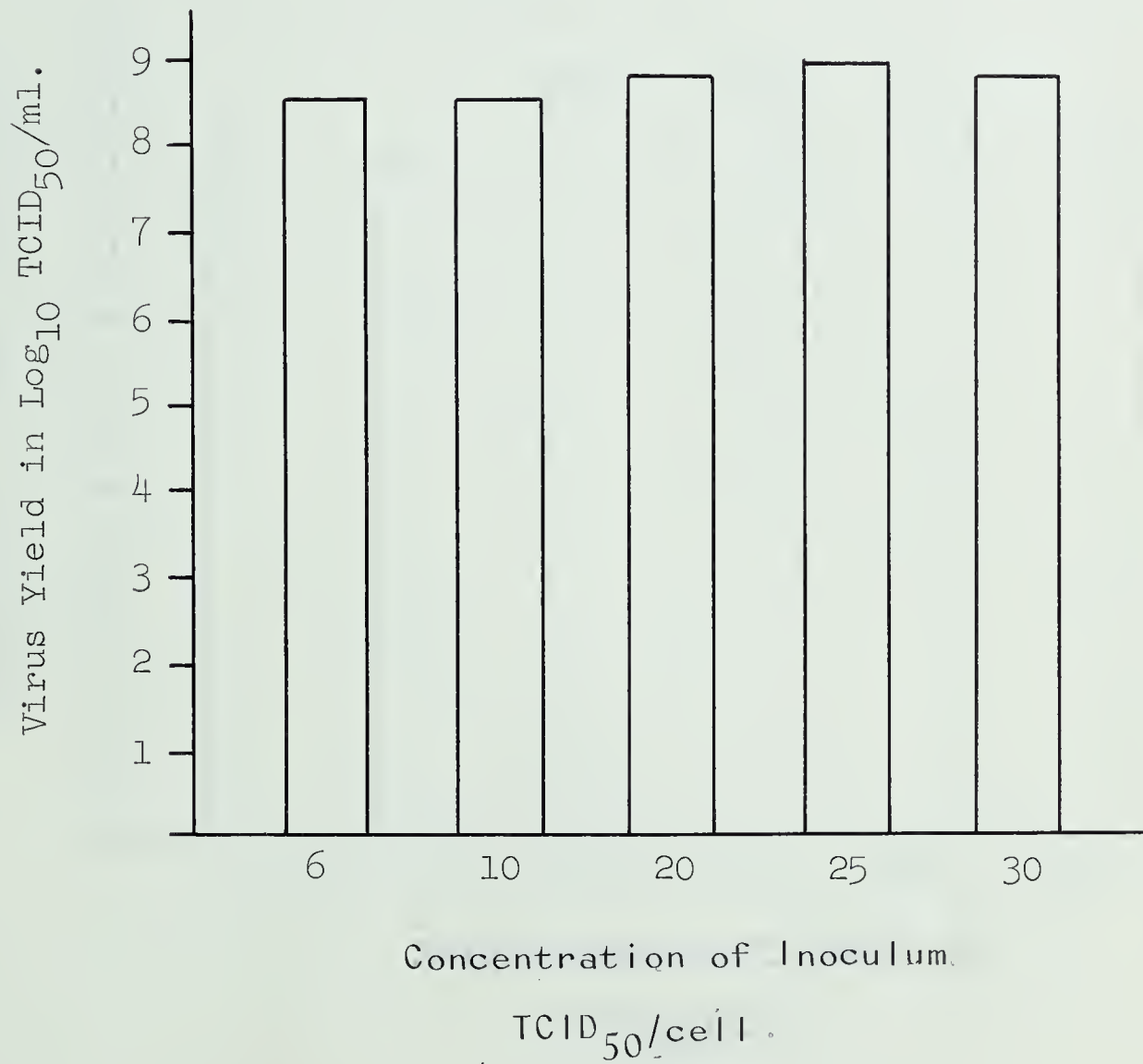






FIGURE #7

Effect of Concentration of Inoculum  
on Production of Toronto A26/61 Virus  
on Monolayers of PDK Cells

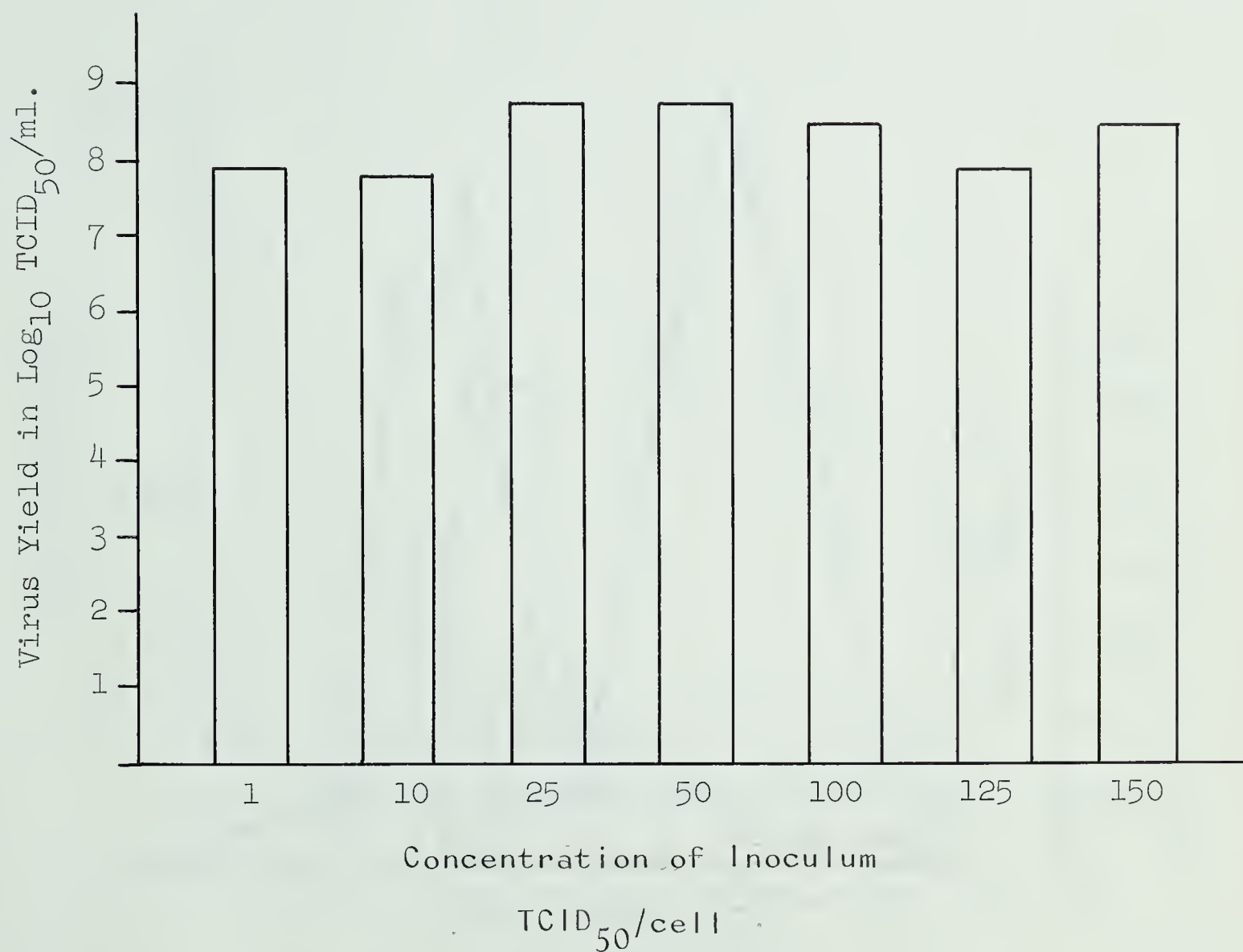
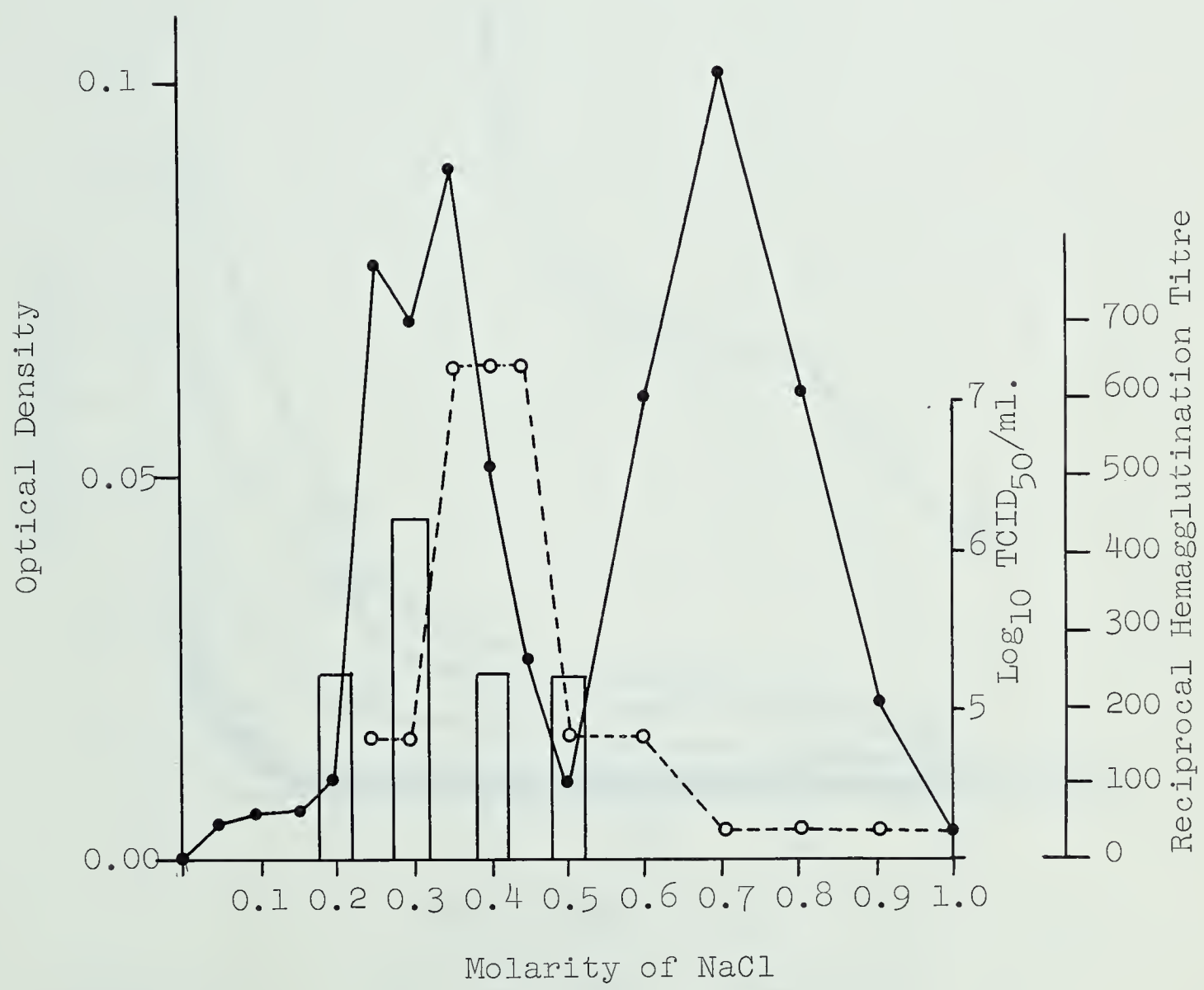




FIGURE #8

Column Chromatography of a Suspension of  
Toronto A26/61 Virus Using  
DEAE-Cellulose



Infectivity

Optical Density ● @ 260 mu.

Hemagglutinin ○

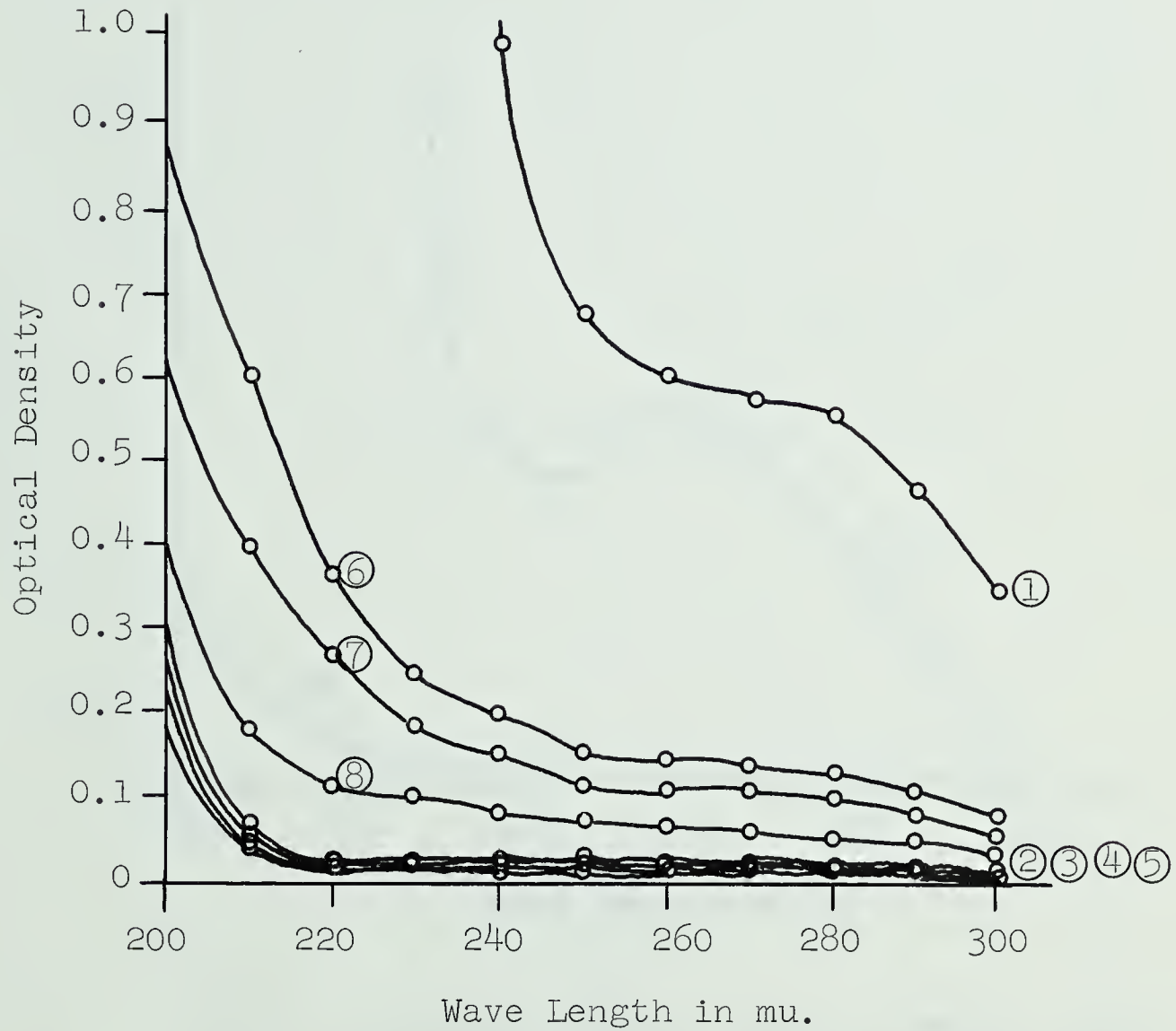


FIGURE #9

Sephadex G-200 Gel

Filtration of Toronto A26/61

Virus Suspension



Fraction 1 contained 69% of infectivity

Fractions 2, 3, 4, 5, contained essentially no infectivity

Fractions 6, 7, 8, collected 2, 4, 6, days after 1 to 5

and exhibited titres of  $10^4$  to  $10^5$

TCID<sub>50</sub>/ml.



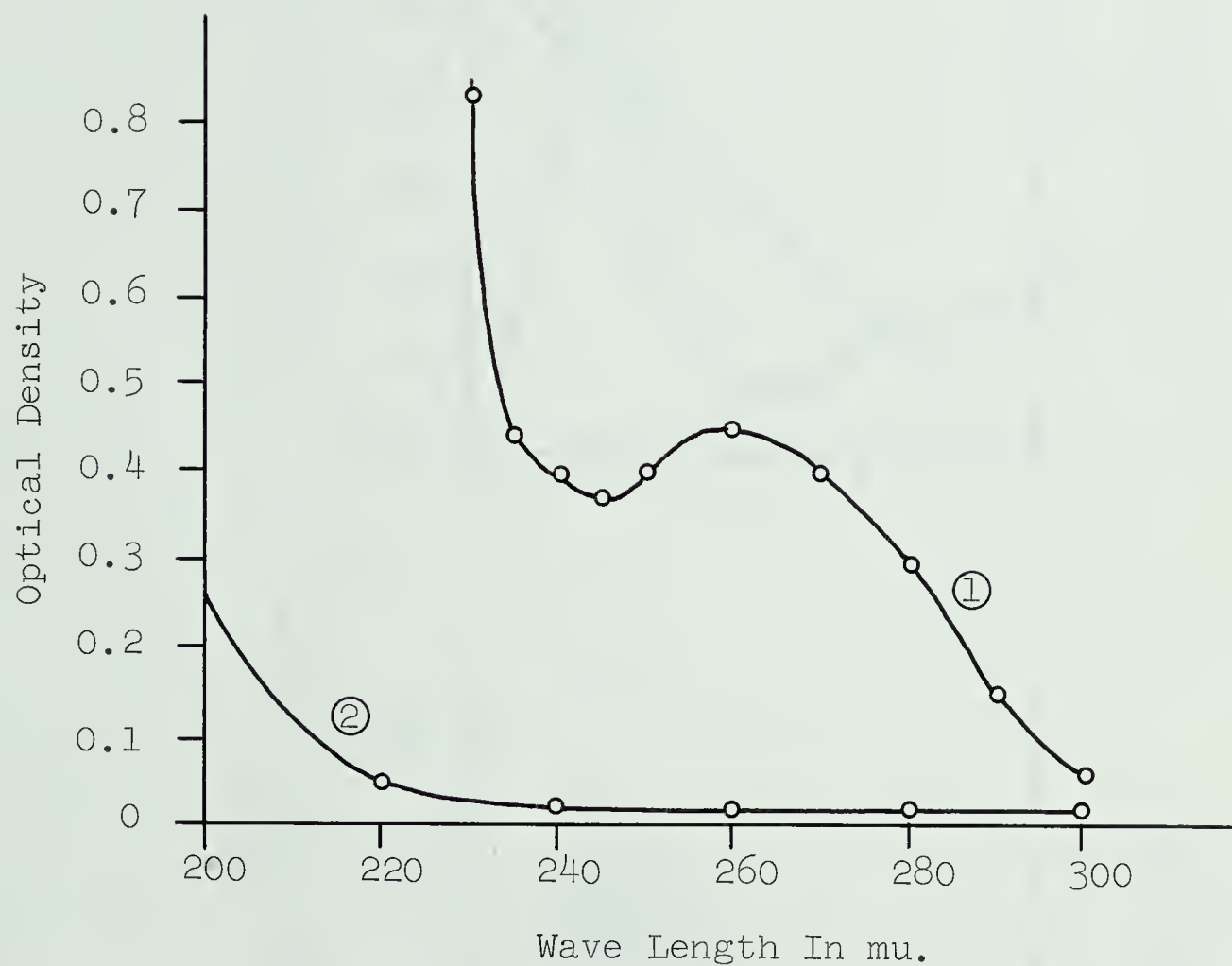


FIGURE #10

Tris Genetron Extraction Of  
DKL Cells Containing Toronto A26/61

Virus

Absorbance Curve

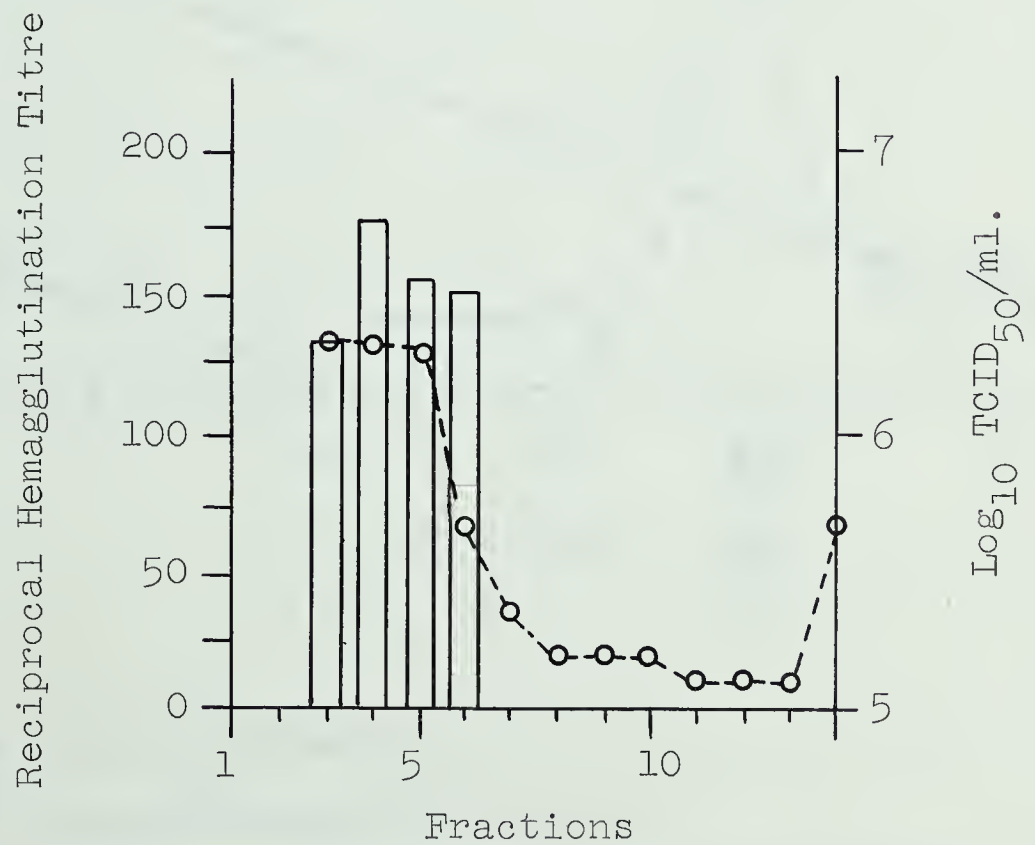
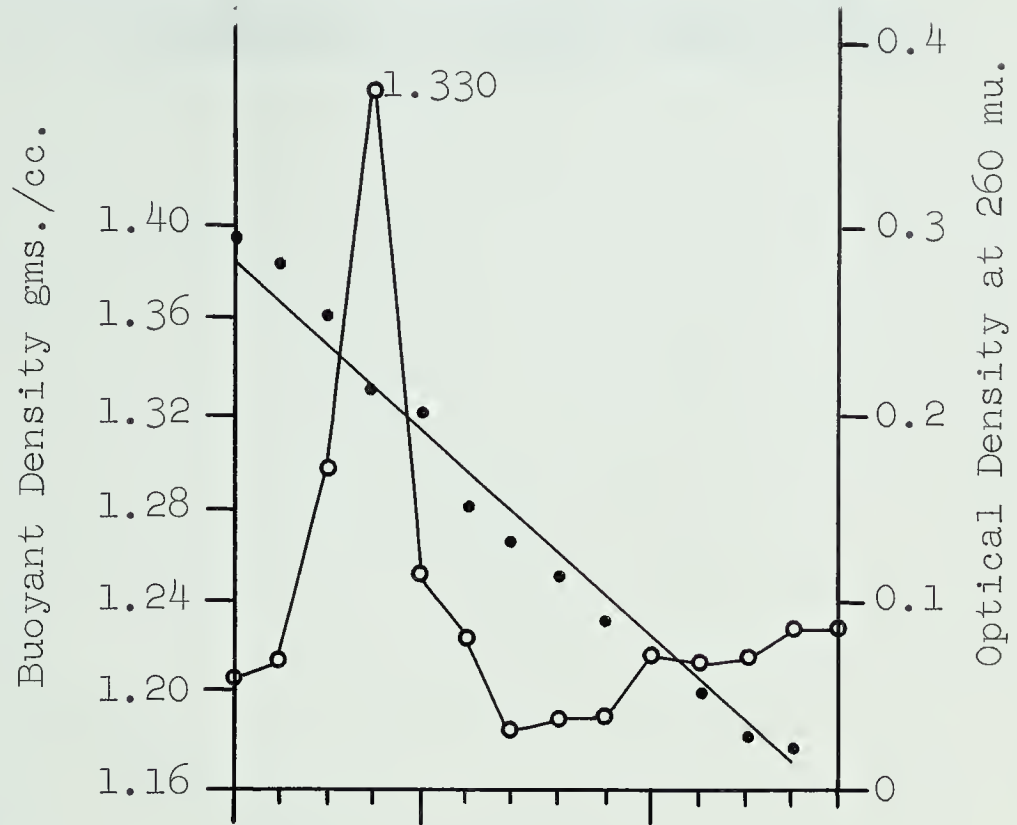



- 1 Prior to treatment with 5.0 ug./ml. DNase & RNase
- 2 After treatment with 5.0 ug./ml. DNase & RNase





FIGURE #11

Buoyant-Density-Gradient Centrifugation  
of a Suspension of Toronto A26/61 Virus



Infectivity 

Hemagglutination 

Optical Density 


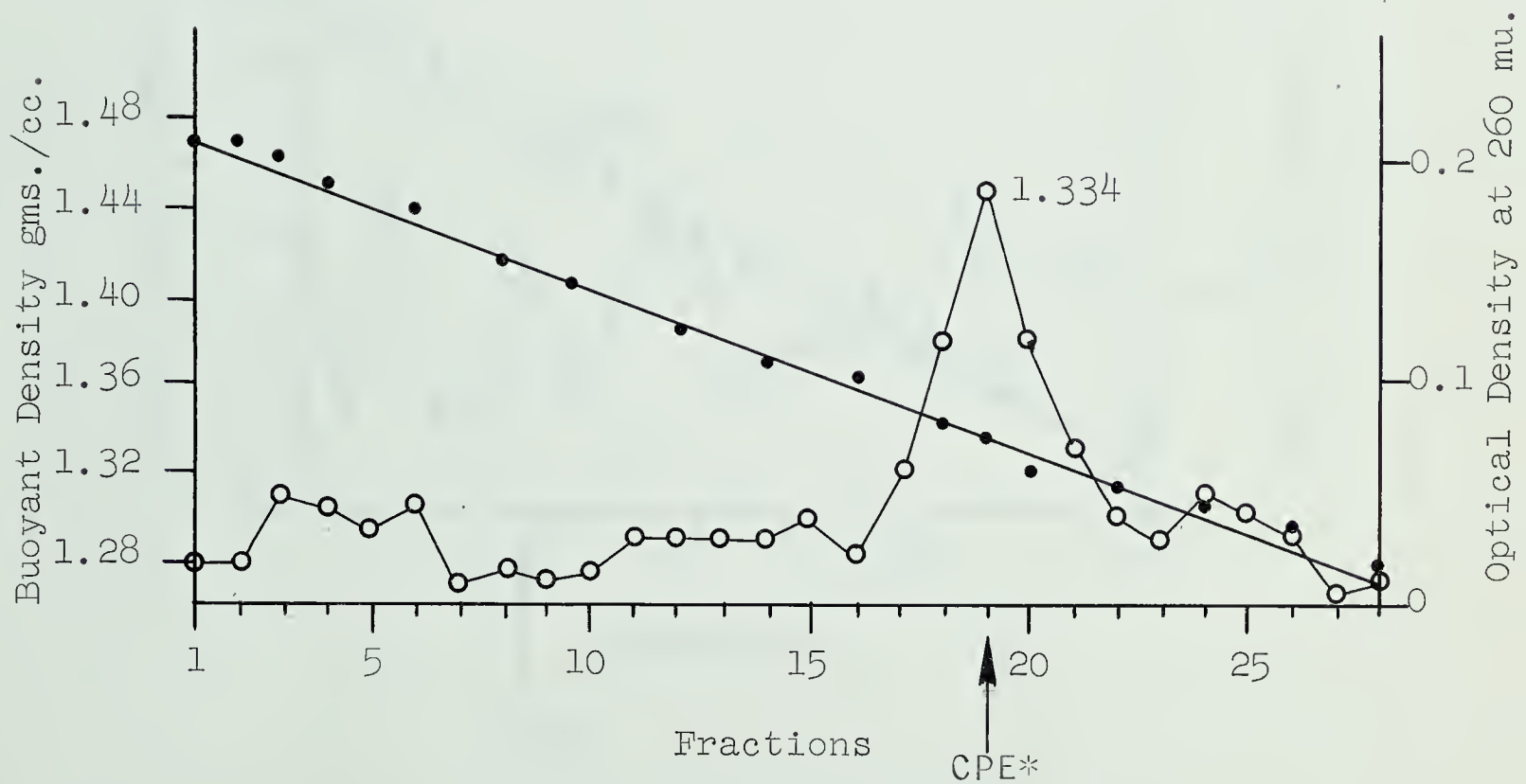
Density Gradient 



FIGURE #12

Buoyant-Density-Gradient Centrifugation  
of a Suspension of Toronto A26/61 Virus

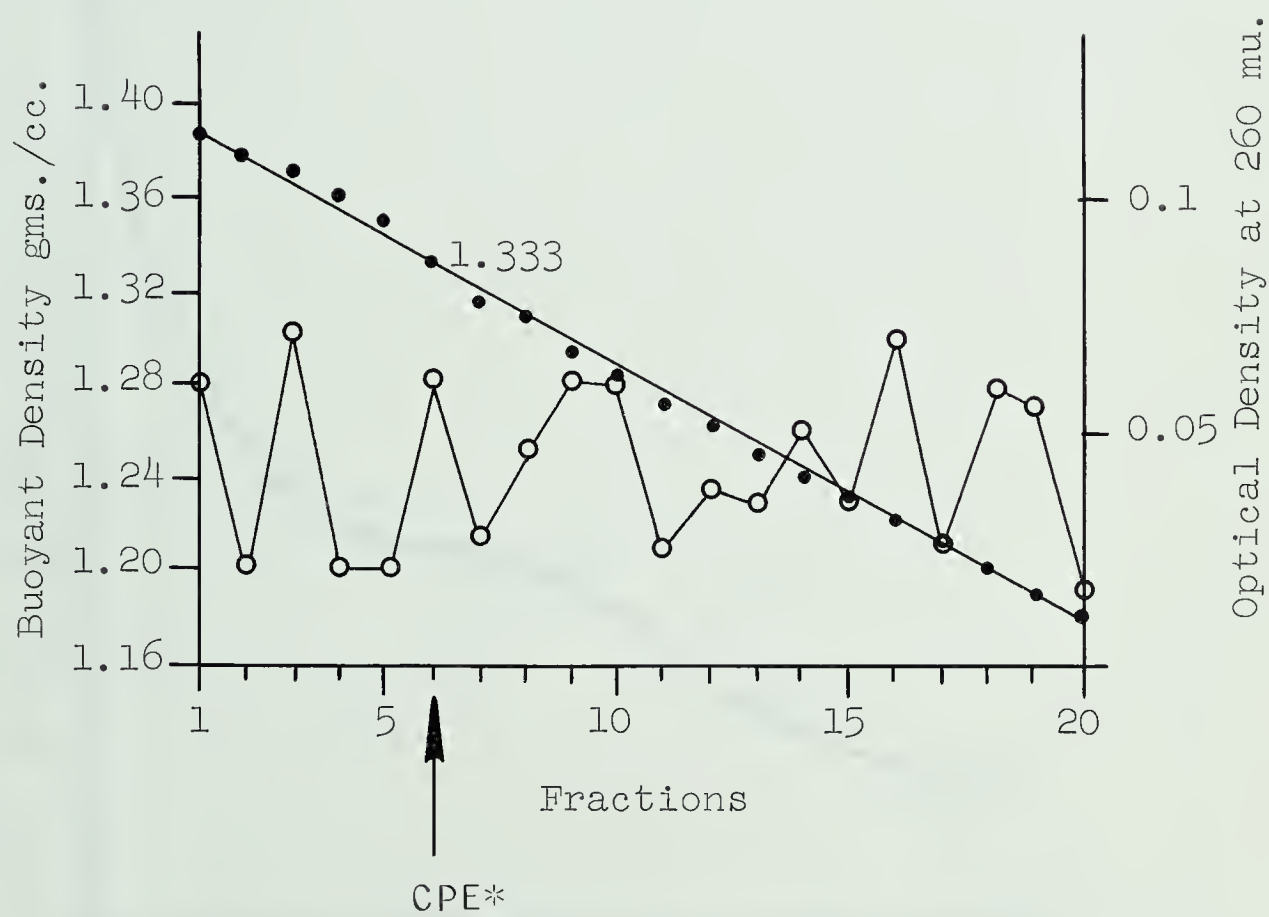


- density gradient
- optical density
- \* maximum cytopathic effect in DKL cells





FIGURE #13  
Buoyant-Density-Gradient Centrifugation  
of a Suspension of ICH Virus



—●—

= density gradient

—○—

= optical density

\*

= maximum cytopathic effect in DKL cells



FIGURE #14

Absorbance Curve of a Toronto A26/61 Virus  
Suspension After Treatment of the Suspension  
with DNase (5.0 ug./ml.)

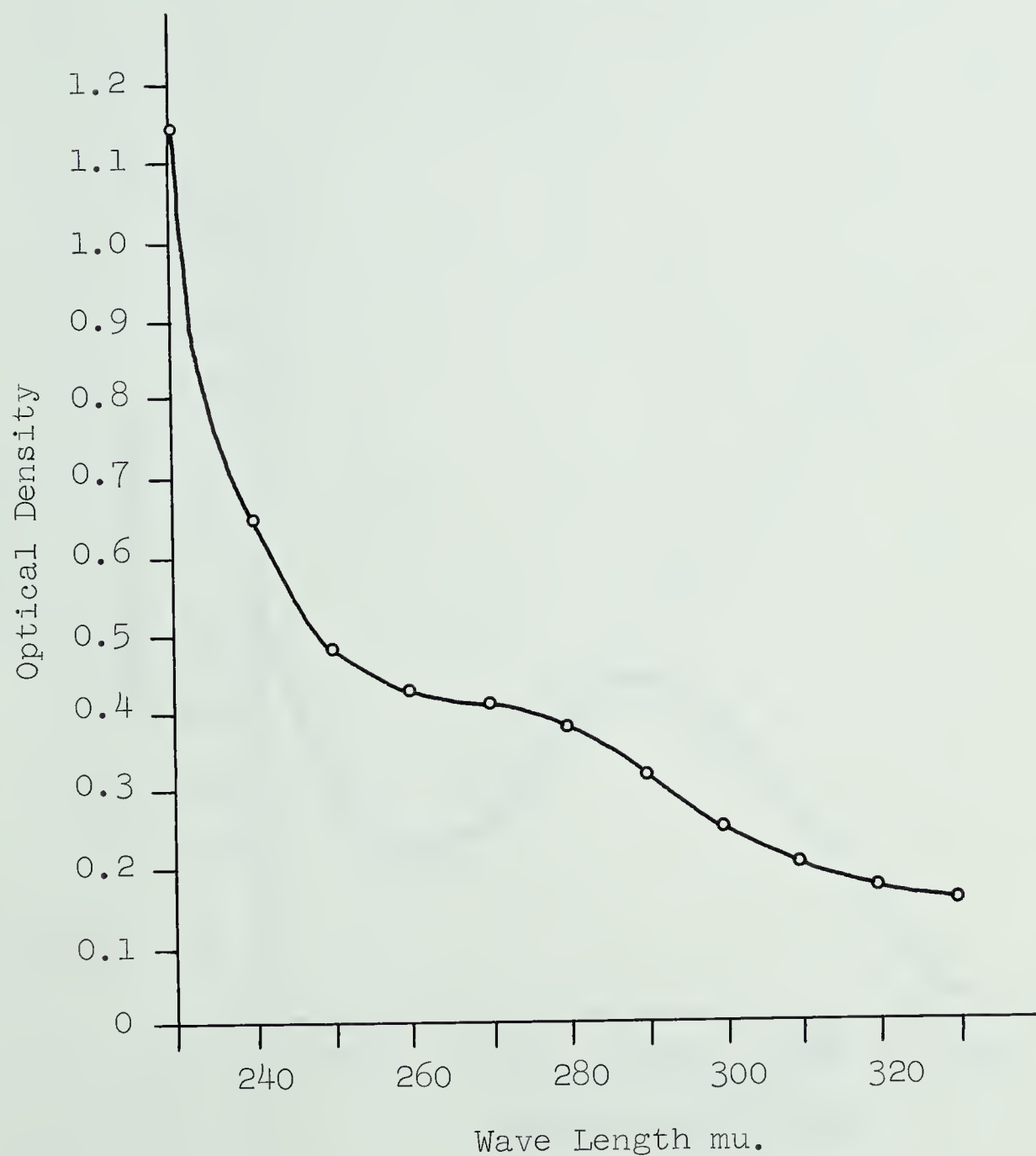




FIGURE #15

Absorbance Curve of DKL Cell DNA  
Solution After Treatment with RNase

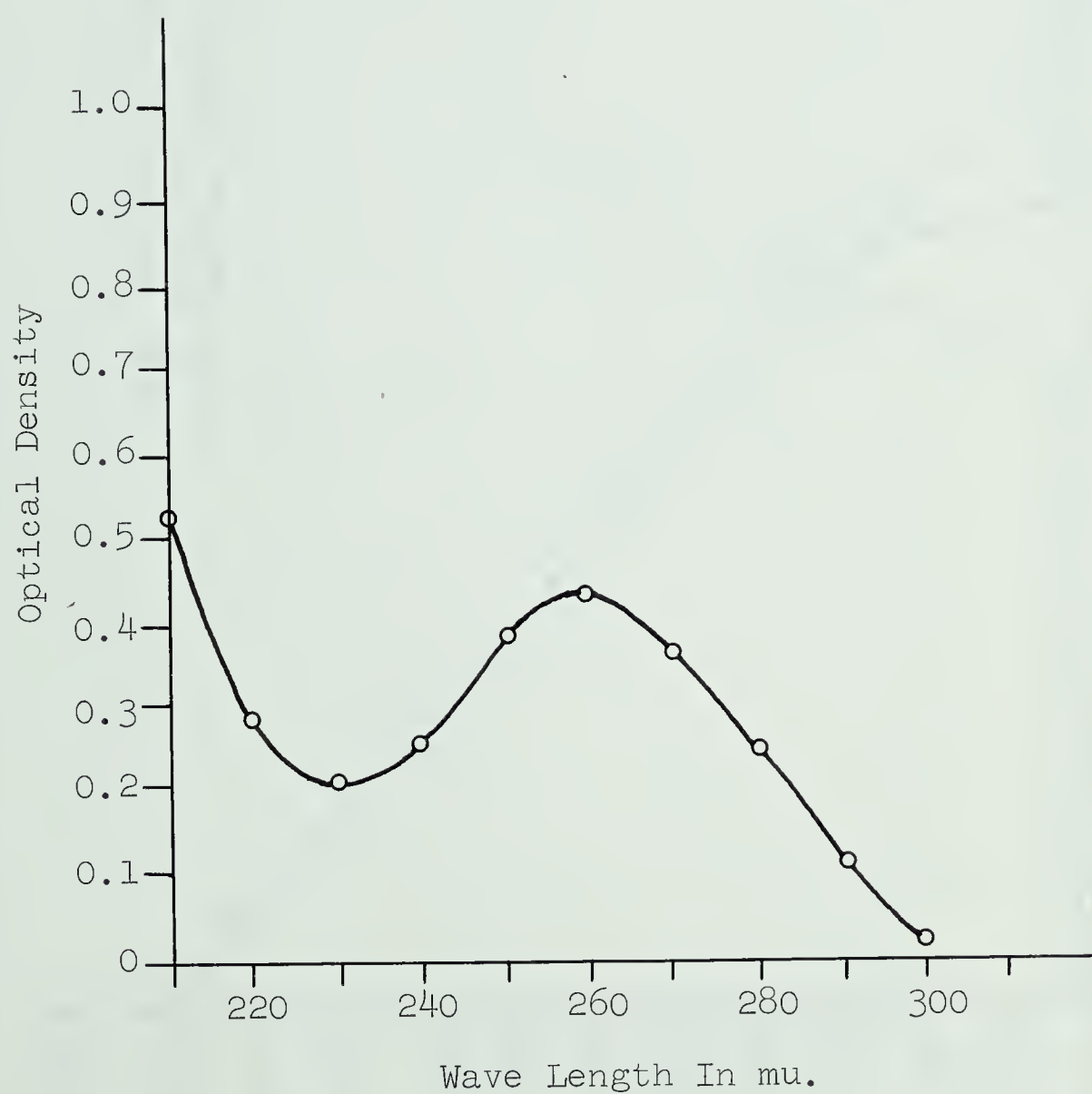






FIGURE #16

Buoyant-Density-Gradient Centrifugation of a Solution of DKL DNA

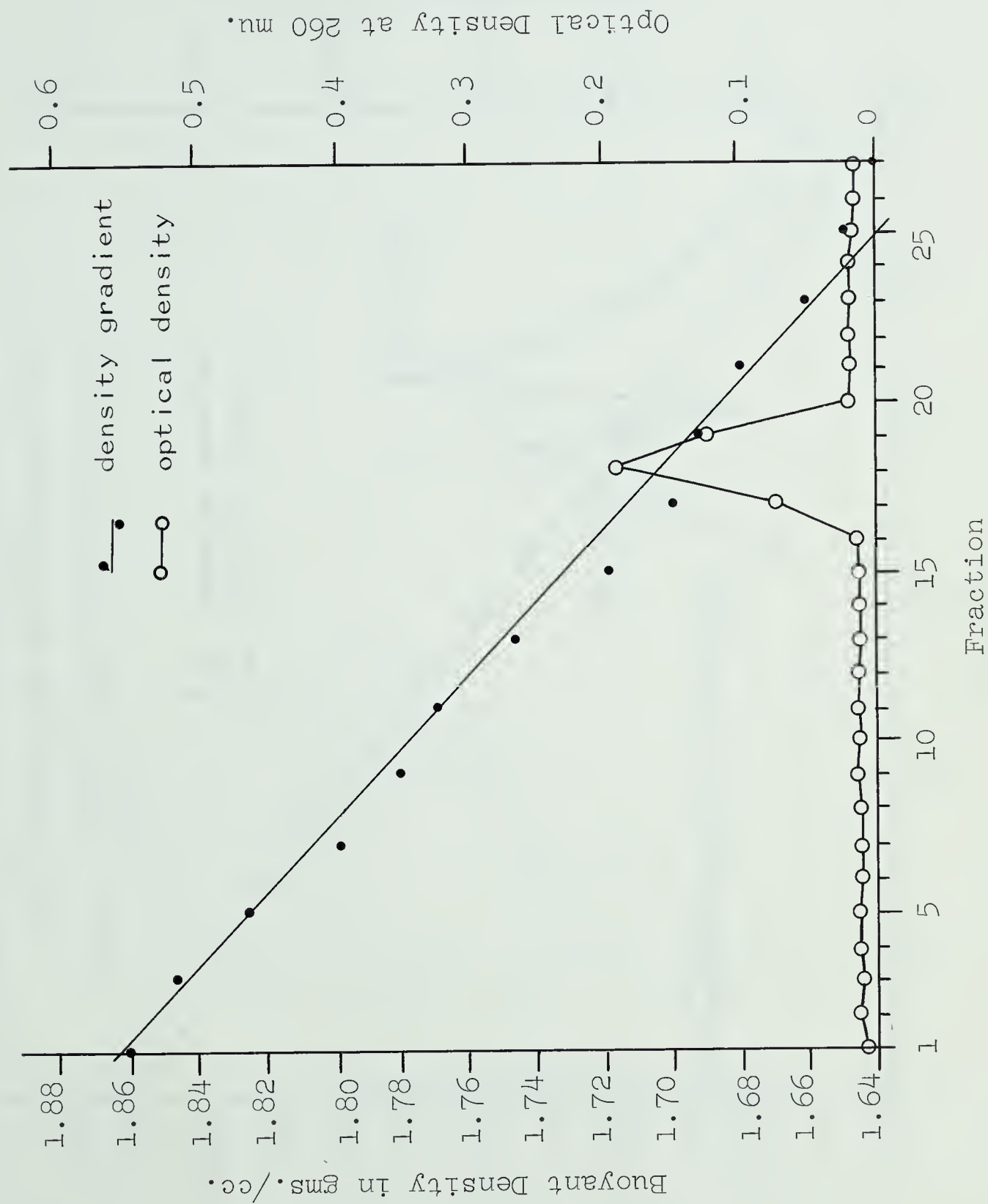




FIGURE #17

Buoyant-Density-Gradient Centrifugation  
of a Solution of DNA Extracted from -  
Toronto A26/61 Virus-Infected DKL Cells

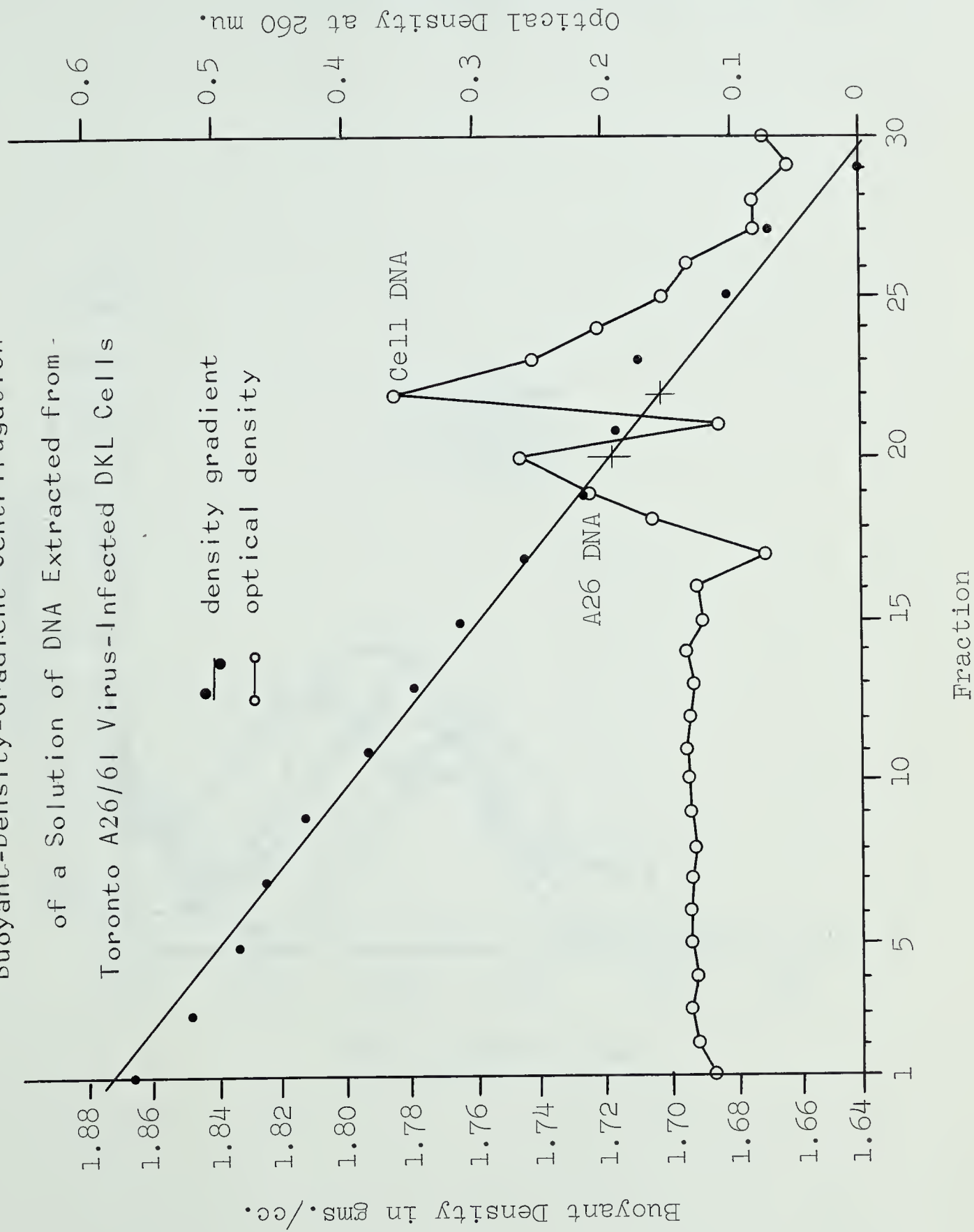




FIGURE #18

Absorbance Curve of Bases  
From Hydrolysed DKL Cell DNA

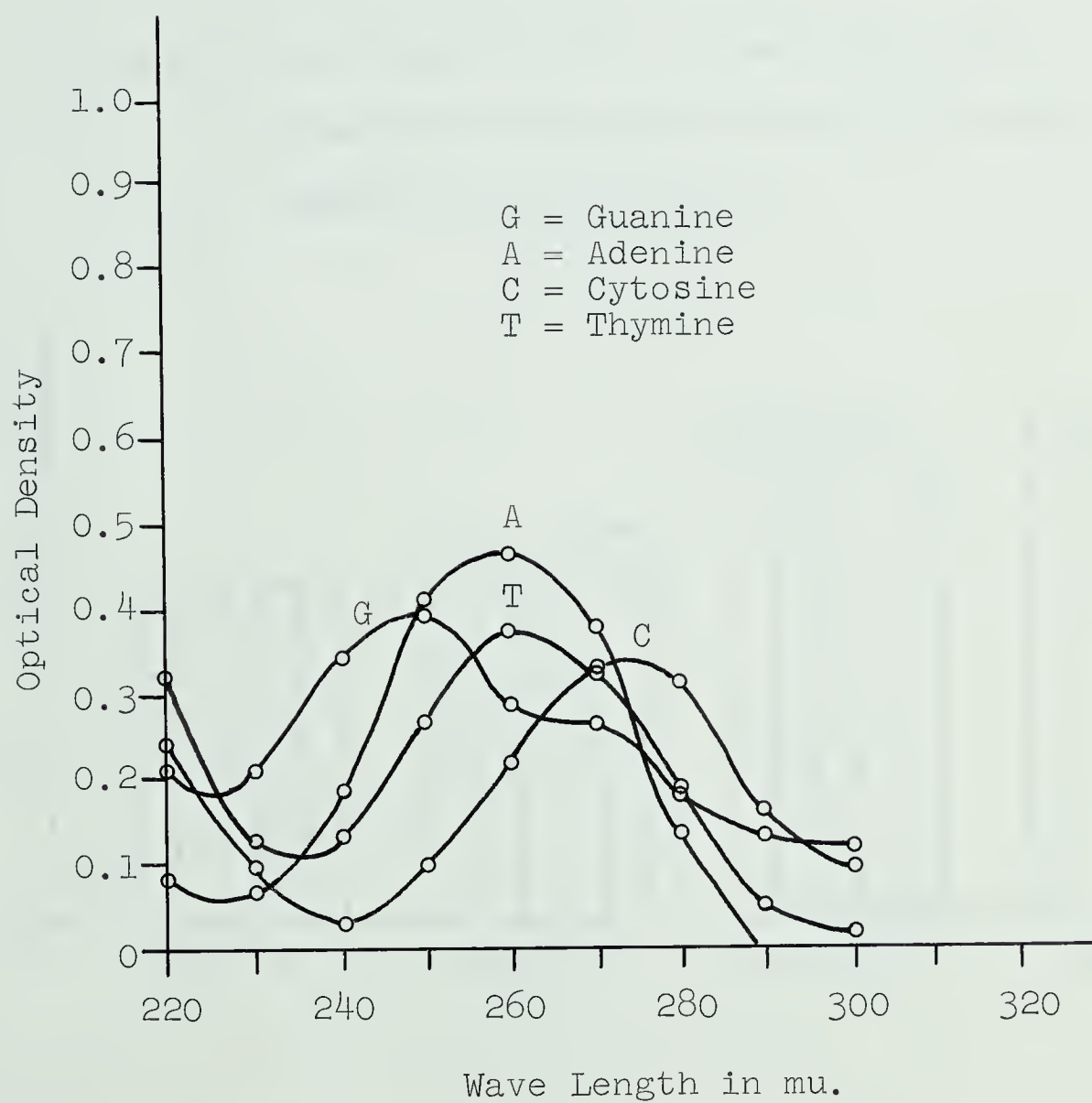






FIGURE #19

Summary of Mole Fraction %'s  
of Bases from Hydrolyzed DKL Cell DNA

Note: Mole fraction %'s derived from five  
separate experiments on five different  
samples of DKL cell DNA

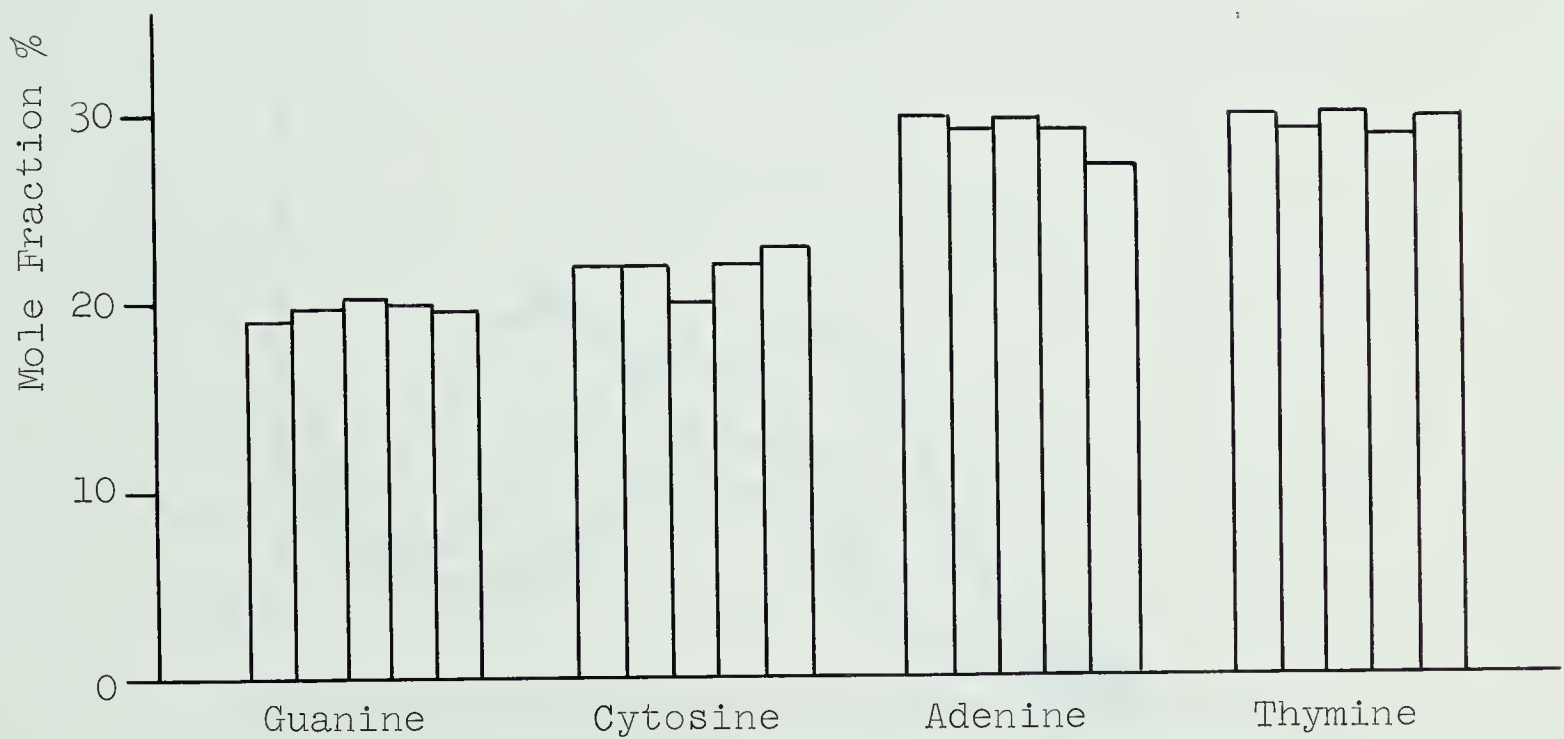




FIGURE #20

Absorbance Curve of Bases From  
Hydrolyzed Toronto A26/61 Virus

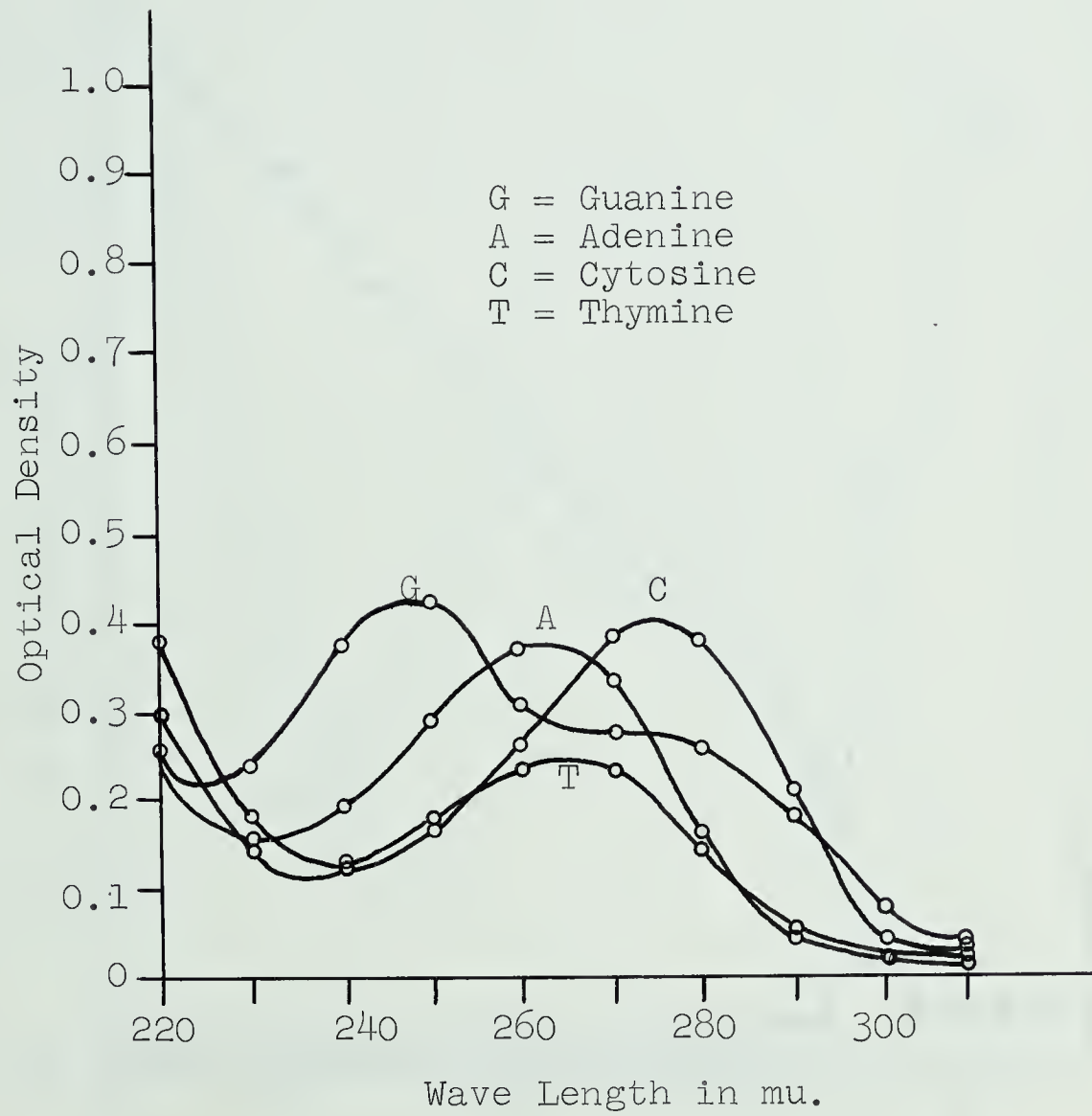
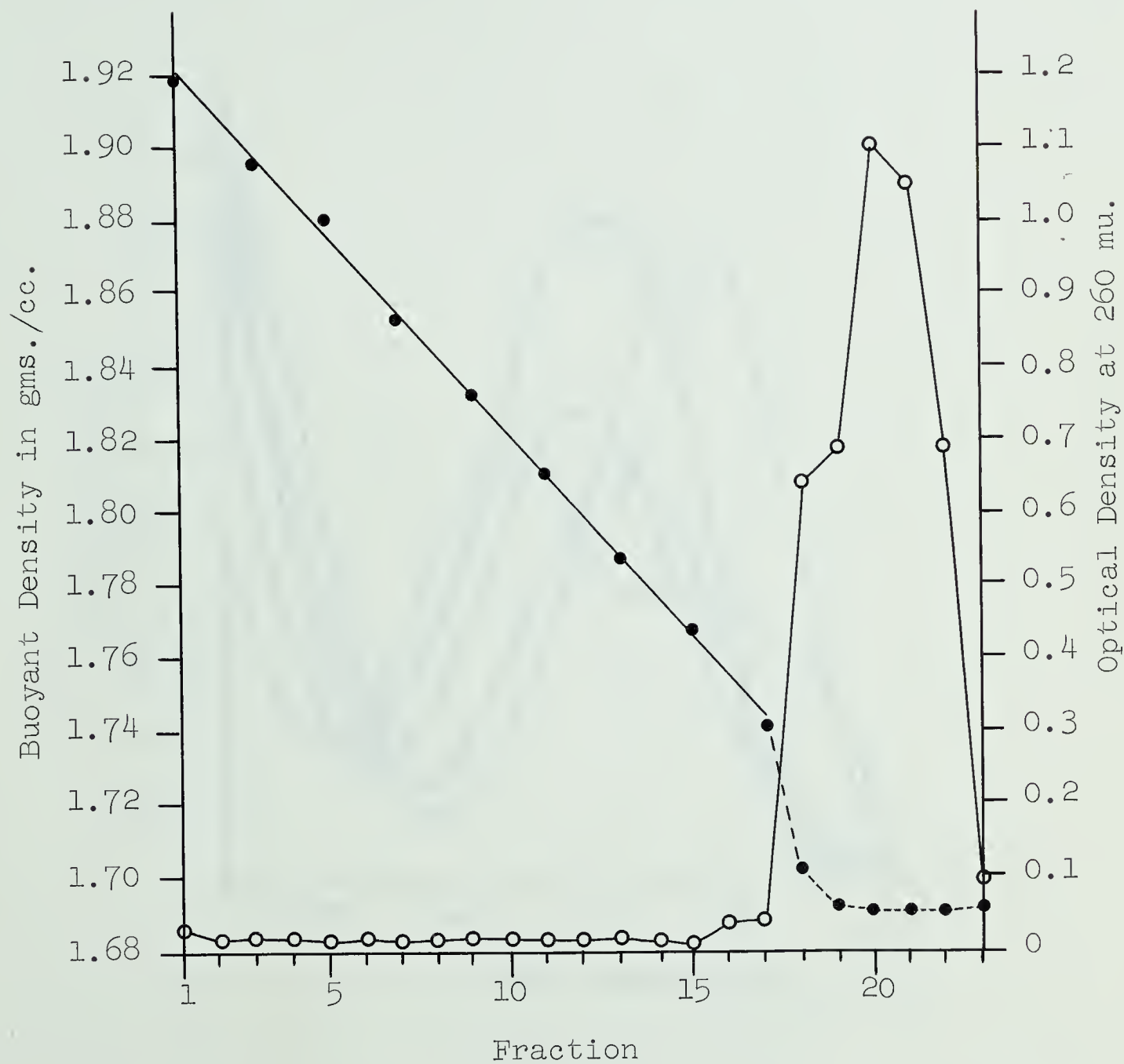




FIGURE #21

Buoyant-Density-Gradient Centrifugation  
of a Solution of Grade A Salmon Sperm DNA



●—● density gradient  
○—○ optical density





FIGURE #22

Absorbance Curve of

G = Guanine  
A = Adenine  
C = Cytosine  
U = Uracil  
T = Thymine

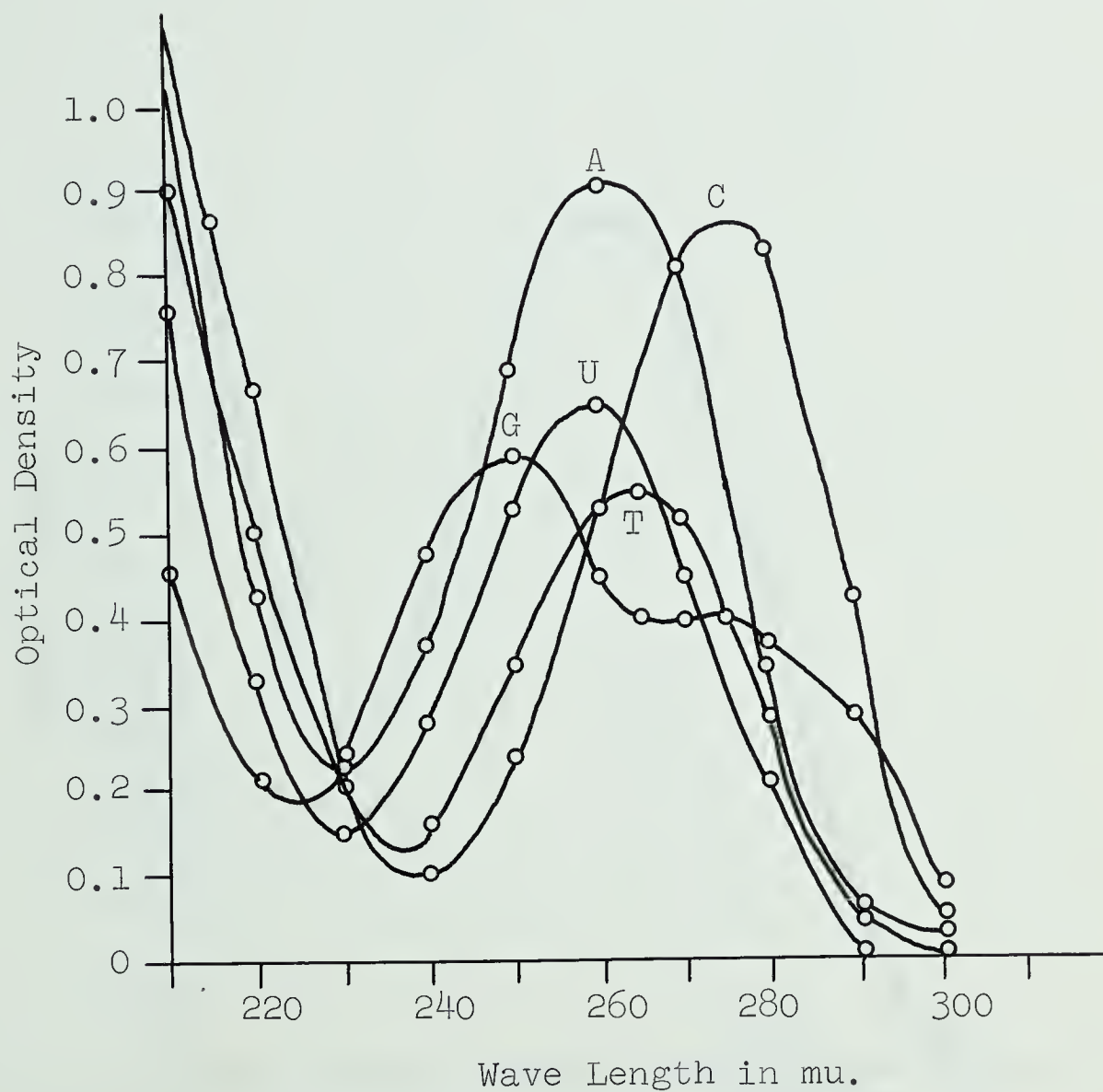




FIGURE #23

Absorbance Curve of  
Salmon Sperm DNA  
(50 ug./ml.)

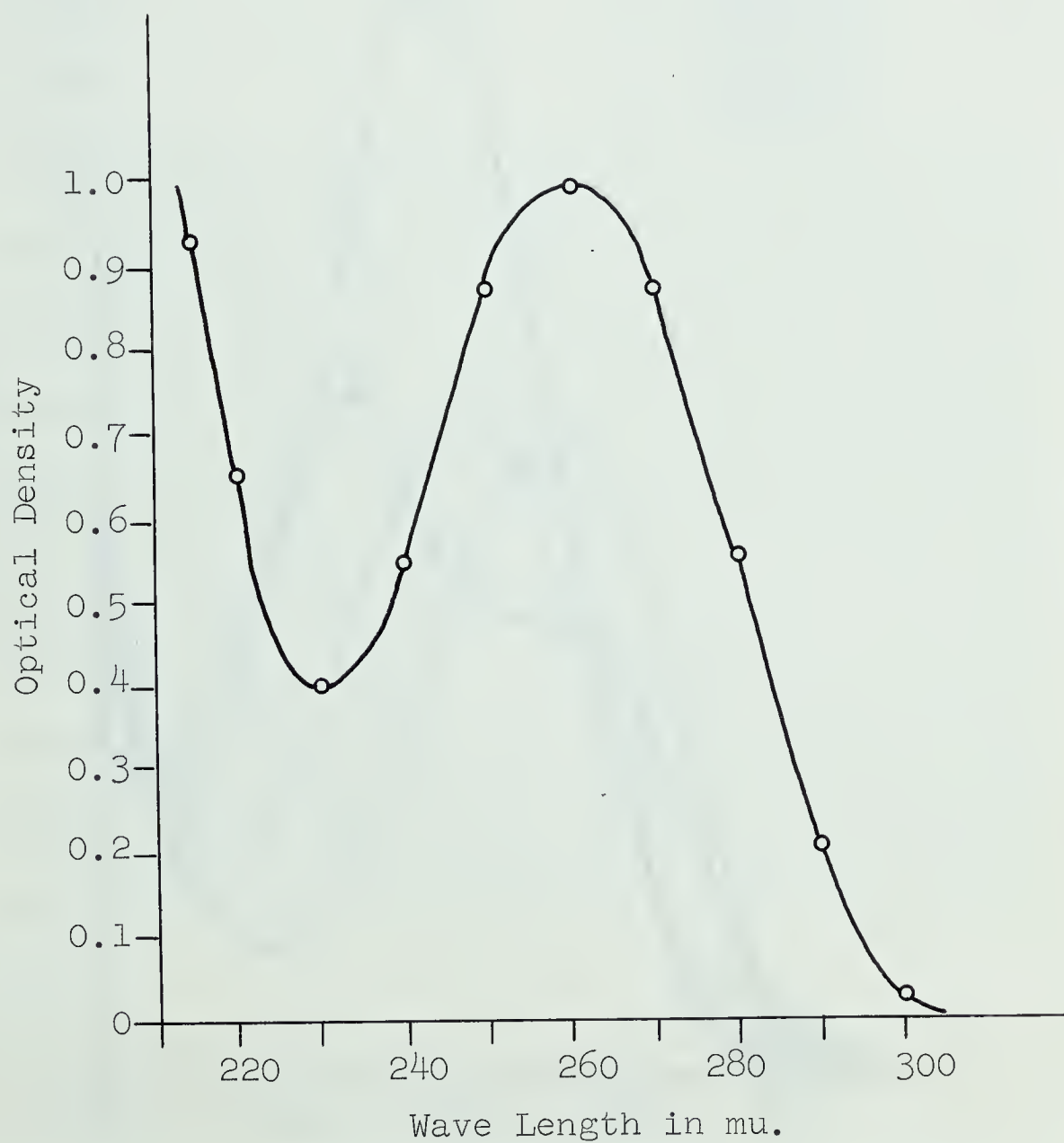
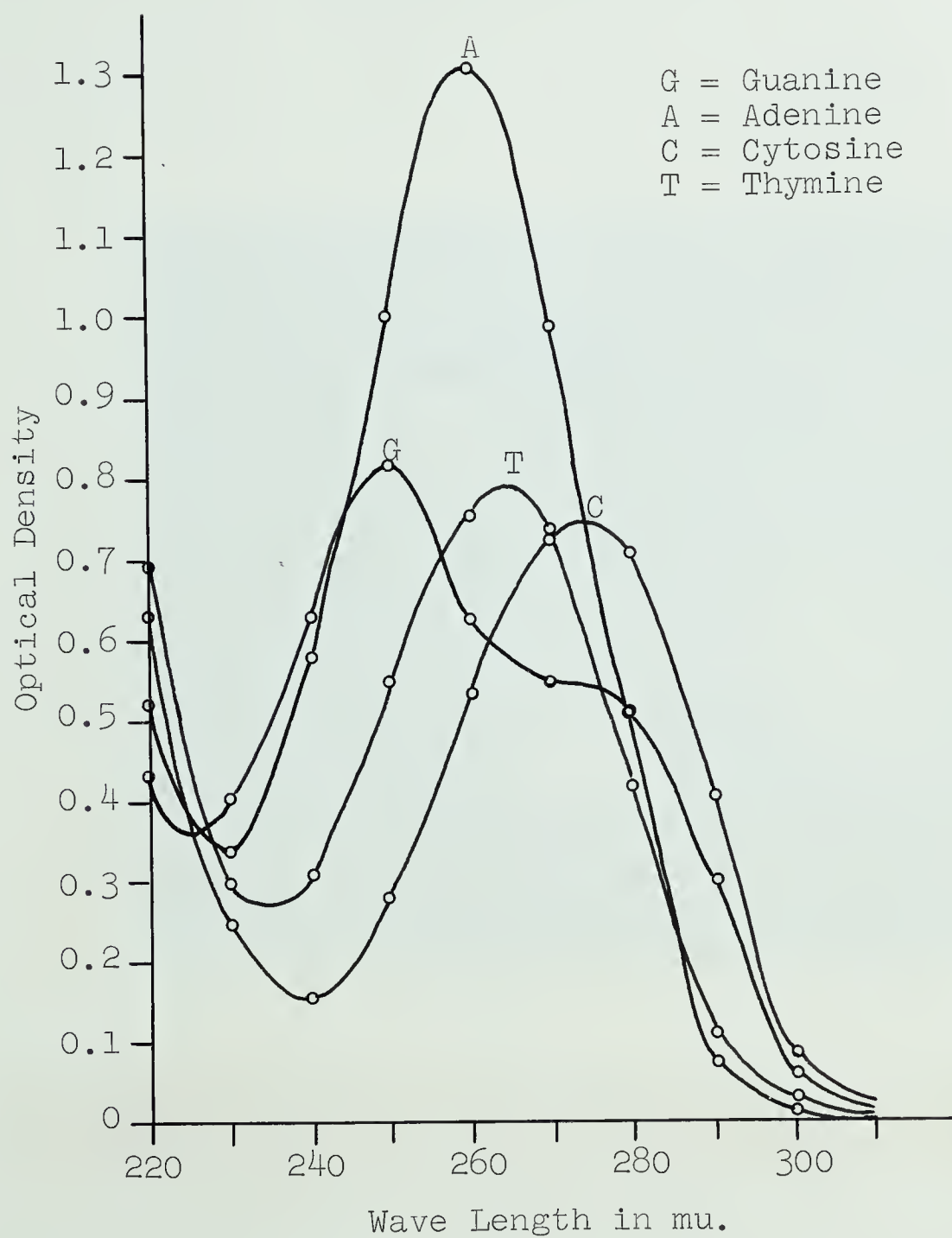




FIGURE #24

Absorbance Curve of Bases from  
Hydrolyzed Salmon Sperm DNA





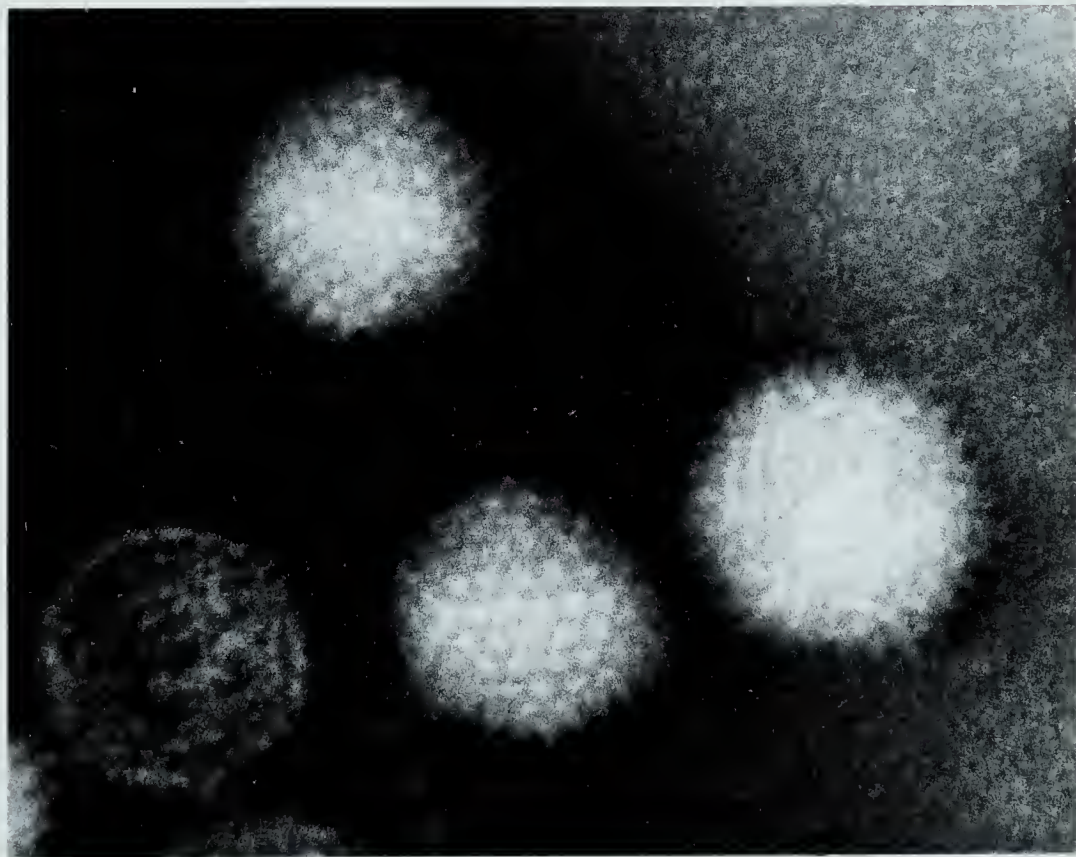


P L A T E S



PLATE #1

ELECTRONMICROGRAPH OF TORONTO A26/61  
AFTER NEGATIVE STAINING WITH PHOSPHOTUNGSTIC ACID  
MAGNIFICATION (400,000 x)



NOTE: TORONTO A26/61 REFERS TO TORONTO A26/61 VIRUS



PLATE #2

PHOTOMICROGRAPH OF UNINFECTED PDK CELLS

STAINED WITH HEMATOXYLIN-EOSIN

MAGNIFICATION (800 x)

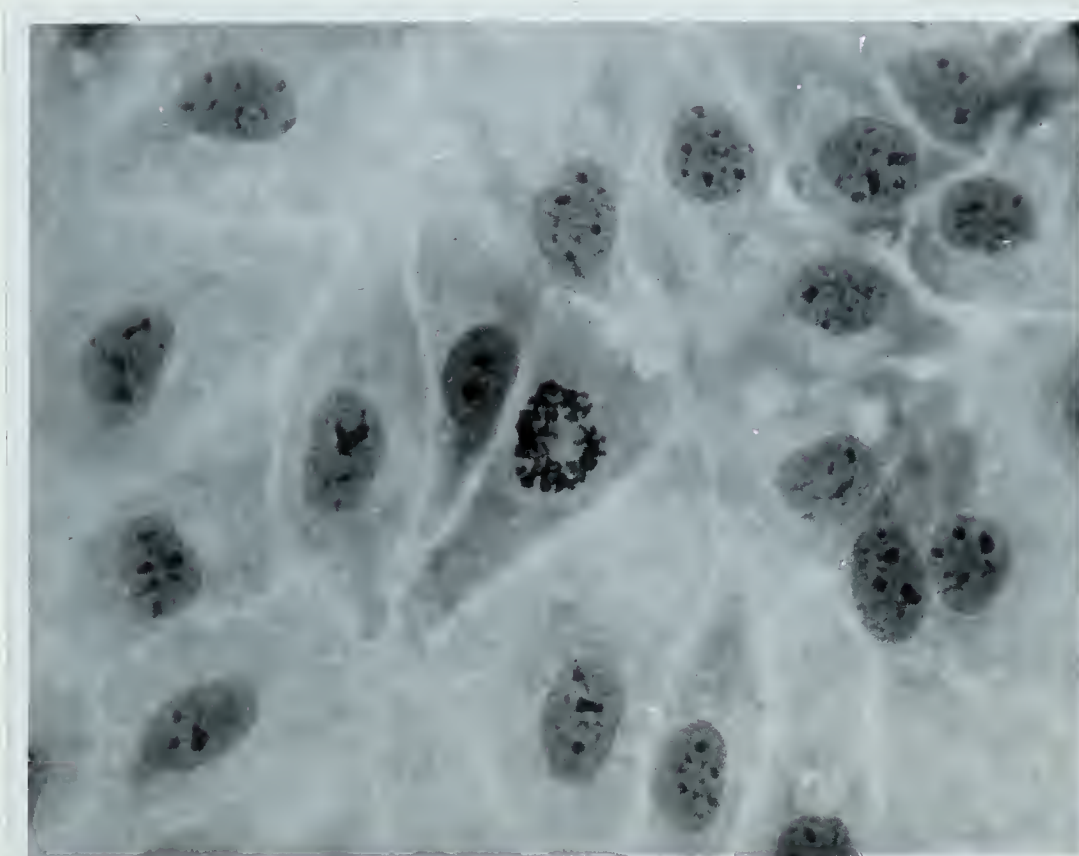
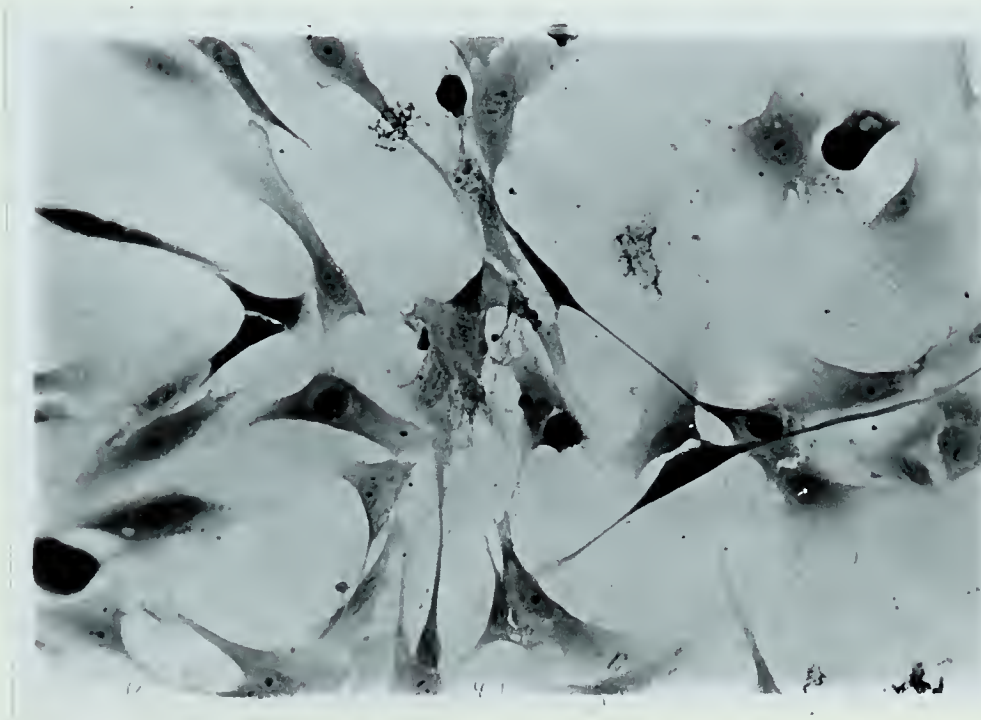






PLATE #3

PHOTOMICROGRAPH OF PDK CELLS  
INFECTED WITH TORONTO A26/61  
STAINED WITH HEMATOXYLIN-EOSIN  
MAGNIFICATION (500 x)



NOTE: TORONTO A26/61 REFERS TO TORONTO A26/61 VIRUS



PLATE #4

PHOTOMICROGRAPH OF PDK CELLS  
SHOWING CYTOPATHIC CHANGES IN NUCLEUS  
DUE TO INFECTION WITH TORONTO A26/61  
STAINED WITH HEMATOXYLIN-EOSIN  
MAGNIFICATION (500 x)



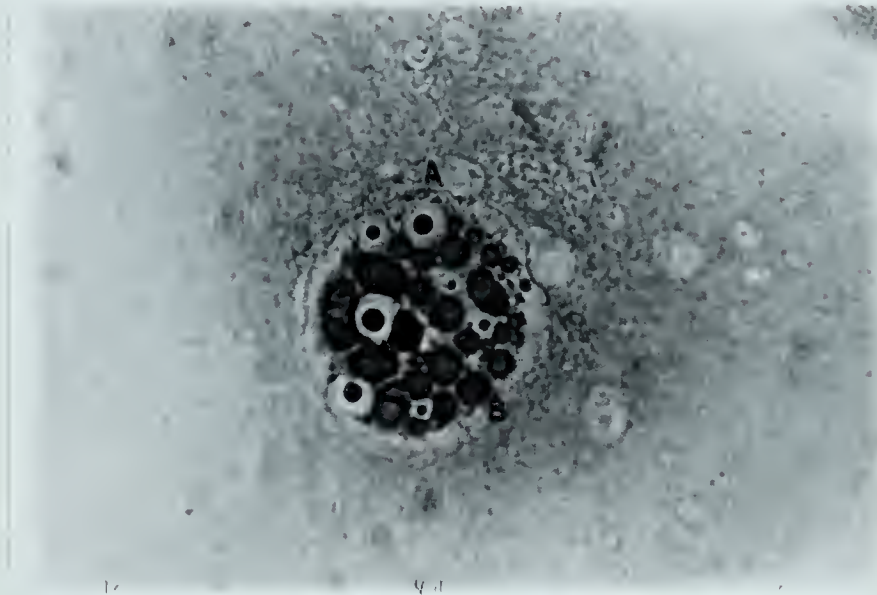
NOTE: TORONTO A26/61 REFERS TO TORONTO A26/61 VIRUS

- A    nucleus in early stages of inclusion formation
- B    basophilic borders becoming evident
- C    ) larger basophilic border
- D    ) faint eosinophilic center
- E    ) vacuolation around nucleus
- F    complete basophilia of nucleus



PLATE #5

PHOTOMICROGRAPH OF NUCLEUS OF  
TORONTO A26/61 INFECTED PDK CELLS  
SHOWING SEVERAL INCLUSIONS  
STAINED WITH HEMATOXYLIN-EOSIN  
MAGNIFICATION (1,000 x)



NOTE: TORONTO A26/61 REFERS TO TORONTO A26/61 VIRUS

A nucleolus

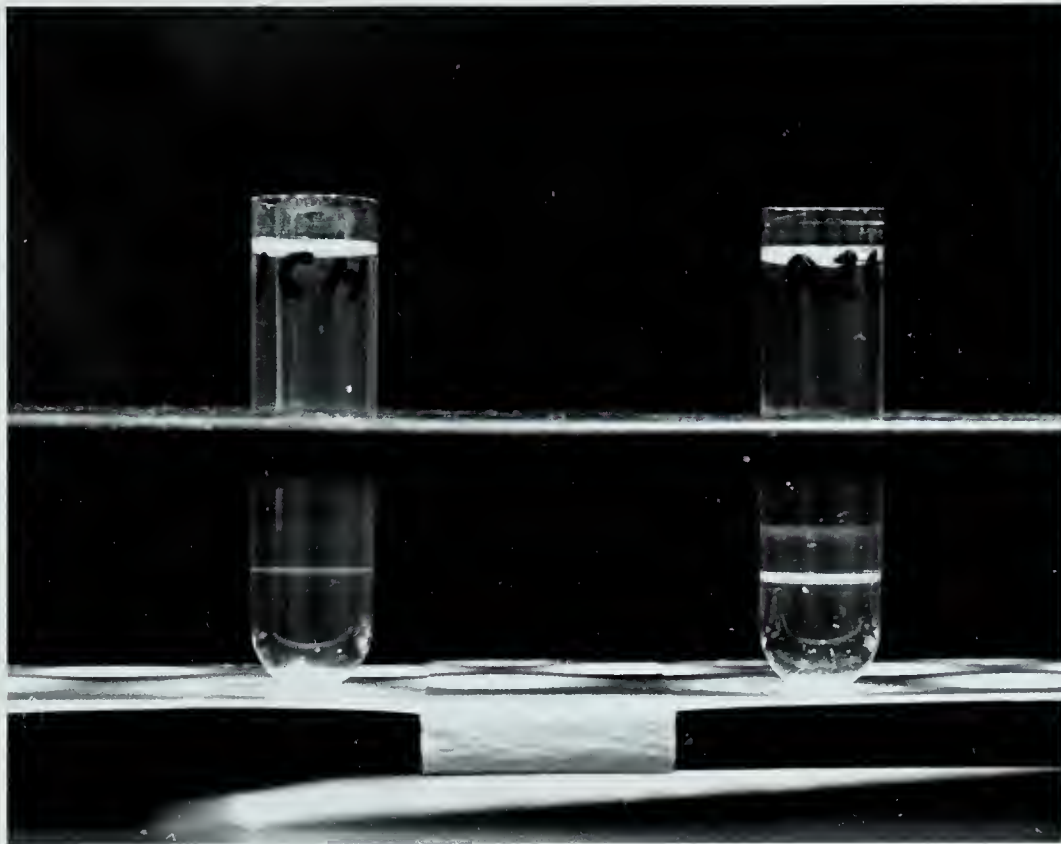
B eosinophilic inclusion with basophilic border





PLATE #6

PHOTOGRAPH (actual size) OF ICH AND TORONTO A26/61  
VIRUSES AFTER BUOYANT DENSITY CENTRIFUGATION  
TO EQUILIBRIUM IN CsCl



Upper Band - incomplete virus

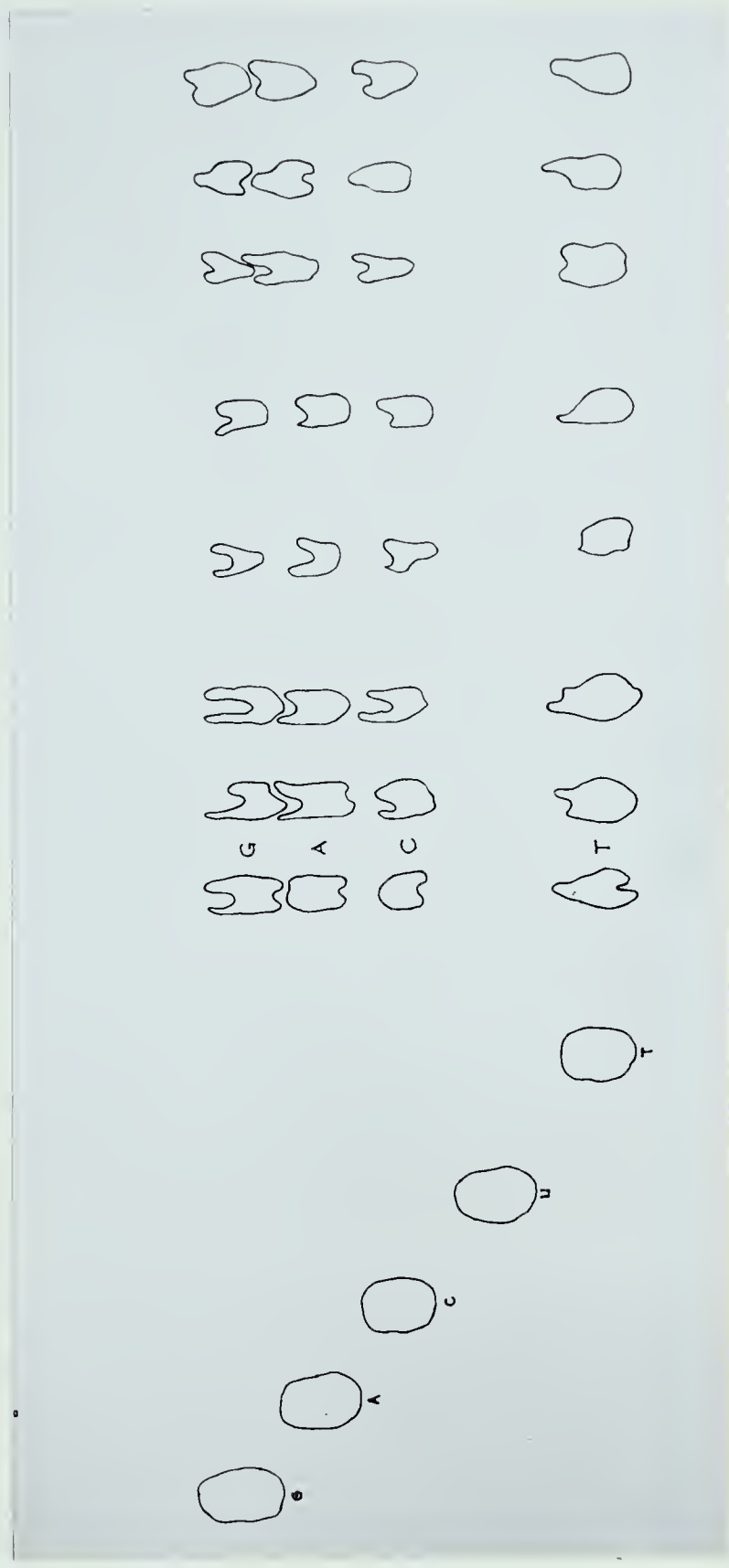
Lower Band - viruses



PLATE #7

COMPARISON OF CHROMATOGRAMS OF THE HYDROLYSED DNA OF TORONTO A26/61  
AND DKL CELL DNA WITH STANDARD BASES AND SALMON SPERM DNA (GRADE A)

STANDARD BASES	SALMON SPERM	TORONTO A26/61	DKL CELLS
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G = Guanine; A = Adenine; C = Cytosine; U = Uracil; T = Thymine

NOTE: TORONTO A26/61 REFERS TO TORONTO A26/61 VIRUS



Ditchfield et al (1962) disclosed that Toronto A26/61 virus would not agglutinate guinea pig or fowl erythrocytes, but that agglutinins were produced against human type "O" erythrocytes. During the course of this investigation this same virus, as well as two strains of infectious canine hepatitis (ICH and HCC) would agglutinate all human erythrocytes of the four main serotypes, A, B, AB, and O. Furthermore, the erythrocytes were agglutinated by the same virus dilution end point irrespective of the serotype. It was also revealed that Toronto A26/61 virus specific antiserum produced in rabbits would inhibit the hemagglutination phenomenon. Whether the hemagglutinin of Toronto A26/61 virus is part of the elementary body or a distinct entity is not known.

The cytopathic changes that take place after the infection of PDK cells with Toronto A26/61 virus seem to be different in sequence than those changes described by Dingle and Ginsberg (1959) in that basophilia first appears on the periphery of the eosinophilic inclusion and subsequently increases toward the centre of the inclusion, gradually reducing the area of eosinophilia until only a very small and faint spot is present. This spot may represent the crystal shown in electron micrographs of thin, sectioned, tissue culture as described by Ditchfield et al (1962).





Inclusion-formation in DKL cells after inoculation with Toronto A26/61 virus was very limited and the limitation was ascribed to the low concentration of infectivity in the inoculum approximately one PFU per two DKL cells. The infectivity was increased to approximately five PFU per cell when PDK cells were used to follow the course of inclusion formation. Inclusions in the PDK cells were much more numerous with approximately 90% of the cell nuclei being affected.

PDK cells were the first cells examined for their ability to produce plaques under the influence of Toronto A26/61 virus. The PDK cells proved to be unsatisfactory for the purpose of assaying the infectivity of Toronto A26/61 virus. Difficulty was experienced in maintaining the viability of PDK cells for ten days -- the minimum time required for plaques induced by Toronto A26/61 virus to be large enough to be observed.

As a result of adenovirus infection, there is an increased synthesis of DNA (Boyer et al, 1957), increased utilization of glucose, and an increased accumulation of certain organic acids which commonly arise from carbohydrate metabolism (Fischer and Ginsberg, 1957). The increased glycolysis and accumulation of organic acids by infected cells cause the fluids of infected cultures to become considerably more acidic than those of uninfected cultures.



These acid conditions were believed responsible for the death of PDK cells. However, plaques were observed (in approximately nine to ten days) when DKL cells were used for the assay of Toronto A26/61 virus. The fact that the DKL cells remained viable for a longer period of time under Toronto A26/61 virus infection might indicate this cell line is not as active metabolically as freshly dispersed primary cell cultures, or, that the buffer systems used in primary cell culture media are not as efficient as the ones used for DKL cell nutritive medium. When different concentrations of nutrient supplements were varied in the nutritive overlay media, little effect was noticed until double the normal concentration of Eagle's essential amino acids was added to the overlay medium. It was mentioned that Bonifas and Schlesinger (1959) found a requirement for arginine which, when added to media caused increased destruction of KB tissue culture cells by adenovirus type 2. This requirement was subsequently proved to be due to PPL0\*contamination of the tissue cultures. Cultures of DKL cells used for the assay of Toronto A26/61 virus were examined for PPL0 contamination as a possible explanation for the plaque enhancement produced by the double normal concentration of Eagle's amino acids. No contamination of DKL cell cultures by PPL0 was discovered.

Green and Pina (1963a) discovered that increasing

\*Rouse et al (1963)





the amino acid concentration of adenovirus type 2 infected KB cells, when the cells were present in a large concentration, ( $10^6$  cells/ml.), resulted in increased virus yields, but that increasing the concentrations of glucose, deoxyribosides, arginine, and calf serum had little effect. They suggest that cultures containing large concentrations of cells may become nutritionally limited. In the case of Toronto A26/61 virus-infected DKL cells, when the concentration of Eagle's amino acids was doubled, the plaque size increased from 1.5 mm. to 3.0 mm. During the course of the plaque assay study, this diameter was only surpassed when protamine sulfate at 75 ug./ml. was incorporated into the overlay medium. Neutral red had a detrimental effect on cells when incorporated in the nutrient agar overlay, (immediately after adsorption of the virus) because the cells were not viable up to ten days. However, when neutral red was added at the end of the incubation period (after ten days) the cells remained viable as evidenced by the uptake of neutral red and plaques produced by the virus were visible for counting.

Kjellen (1961) described the lethal effects produced by neutral red when adenovirus-(type 4 and 5) infected bone marrow (MAS) cells were used.

DEAE-dextran, in a concentration of 100 to 150 ug./ml., had an enhancing effect on plaque formation. When





DEAE-dextran was omitted from the overlay material, plaques were seldom larger than 1 to 1.5 mm. in diameter. When DEAE-dextran was added to the overlay, plaque sizes varied from 1.5 to 2.5 mm. in diameter at optimum concentration: (i.e. 100 to 150 ug./ml.). Protamine sulfate had a slightly greater enhancing effect on plaque size than did the DEAE-dextran. When 75 ug./ml. of protamine sulfate was added to overlay material a plaque size of 4 mm. in diameter was observed. If the assumptions on the part of Liebhaver and Takemoto (1961), Liebhaver and Takemoto (1963), Colter et al (1964), Campbell and Colter (1965) are correct (that sulfated polysaccharide inhibitors, released from agar upon autoclaving, are responsible for diminution of plaque size and that DEAE-dextran and protamine sulfate counteract the effects of these inhibitors in various ways) then it would appear that agar inhibitors are present in the Toronto A26/61 virus-DKL cell assay system and that various concentrations of DEAE-dextran or protamine sulfate are indeed counteracting this inhibition.

Plaque development in tissue culture dishes in the CO<sub>2</sub> incubator gave reproducibly better results. The pH of the tissue-virus system can be maintained near



neutral for a much longer period of time than is possible in stoppered three-ounce bottles, which tended to show acid conditions in about three to five days after application of the overlay.

While the different additives used appear to enhance the formation of plaques, pH is a very important factor. If the pH can be maintained near neutral for ten days with the overlay, plaques appear before the death of the cells.

Vogt et al, (1957) state that the cytopathic effect of variants of poliovirus characterized by low pathogenicity for monkeys is delayed in slightly acid media as indicated by the slower formation of plaques in agar overlay cultures of monkey renal cells.

Barron and Karzon (1957) state that the cytopathic changes induced by certain strains of Echo and Coxsackie viruses are also depressed or delayed when the pH of the medium is allowed to become acid.

The omission of glucose from the growth media should be examined to see if it would control these acid conditions to a more significant degree.

Kjellen (1961) in his studies with adenovirus type 4 and 5 stated that a continued increase in plaque titre was observed even up to 28 days on very good monolayers. Virus infected DKL cell monolayers in a few



instances showed approximately a 10% increase in visible plaques after being kept to the limit of their viability (15 days).

Reproducibility of results with regard to the plaque assay method depended to a large degree on maintaining pH near neutral during the period of assay, using monolayers of the same age and phase of growth during the titration. A series of experiments should be conducted where cell sheets of different ages are assayed in an endeavor to obtain the best growth phase for the plaque assay of Toronto A26/61 virus.

Suspension cultures of DKL cells for the propagation of Toronto A26/61 virus were unsatisfactory. The highest yield of virus was observed at a cell concentration of 750,000 cells/ml., but this yield was still very low as compared with yields obtained from monolayer cultures infected with Toronto A26/61 virus. The yield obtained from the preparation containing 750,000 cells/ml. was  $1.6 \times 10^5$  PFU/ml. or a yield of approximately 2 PFU for every 10 cells as compared with an inoculum concentration of 1 TCID<sub>50</sub>/cell.

Yields for monolayer cultures varied from  $1 \times 10^7$  to  $1 \times 10^8$  TCID<sub>50</sub>/ml., or approximately 4 to 8 TCID<sub>50</sub>/cell using a similar inoculum concentration of 1 TCID<sub>50</sub>/cell.

Green and Pina (1963a) obtained yields of human







adenovirus type 2 from suspension cultures of KB cells in titres of  $1 \times 10^9$  PFU/ml. They achieved highest titres when cell densities were in the range of 100,000 - 200,000 cells/ml. Their multiplicities of infection, however, were of the order of 100 PFU/cell which is in contrast to the Toronto A26/61-DKL cell system where concentration of infectivity was approximately 1 TCID<sub>50</sub>/cell.

Because of the poor yield obtained using suspension cultures, Toronto A26/61 virus was propagated in monolayer cultures of DKL cells in Roux bottles.

Variation in the concentration of infectivity of the inoculum (between 1 TCID<sub>50</sub>/cell to 150 TCID<sub>50</sub>/cell) had little effect on the final yield of Toronto A26/61 virus. DKL cells proved to be as efficient as PDK cells for the propagation of Toronto A26/61 virus. Because DKL cell cultures are much more easily prepared than primary cultures, DKL cells were utilized for the subsequent propagation of Toronto A26/61 virus.

Differential centrifugation combined with buoyant-density-gradient centrifugation was the method of choice for the concentration of Toronto A26/61 virus. The inactivation



of the virus evidenced by loss of infectivity during centrifugation was probably due to clumping of the virus. Infectivity was, therefore, not used as a criterion in assessing the quantity of virus obtained, but rather the optical density at 260 mu. displayed by the virus suspension. The primary concern was to obtain optimum amounts of viral nucleic acid for subsequent chemical studies. Attempts to concentrate Toronto A26/61 virus using a chromatographic column containing DEAE-cellulose did not yield satisfactory results. Optical density readings at 260 mu. on the collected fractions were very low (less than 0.1). Only 34% of the original infectivity was recovered and only 16.7% of the hemagglutinin was recovered. Toronto A26/61 virus either adheres too tenaciously to the DEAE-cellulose column, or the virus particles are sufficiently altered to lose infectivity.

The infectivity and hemagglutinin were eluted from the column in a range between 0.2 M. to 0.5 M. sodium chloride. This elution pattern agrees with work completed by Yamamoto (in press) and is in contrast to studies conducted by Green and Pina (1963a) on human adenovirus types 2 and 4 where the majority of the infectivity was eluted between 0.5 M. to 1.0 M. sodium chloride. They recovered approximately 20% of the original infectivity. In other studies on human adenovirus type 2, Philipson (1960), using



DEAE-cellulose, eluted 15% of the virus at 0.5 M. sodium chloride and 20% at 0.75 M. sodium chloride, thus recovering only 35% of the original infectivity.

It will be noticed in Figure #8 that another peak was eluted at approximately 0.7 M. NaCl but no infectivity was correlated with this peak.

Studies by Commoner et al (1956) concerning correlations between biological activities and specified chemical attributes of the tobacco mosaic virus agent when chromatographed on Ecteola-SF cellulose showed that the intact agent was eluted with 0.04 M. - 0.10 M. NaCl and nucleoprotein was eluted with 0.06 M. - 0.08 M. NaCl.

Tausser and Creaser (1957) showed the elution pattern of infectious  $T_2$  bacteriophage particles was 0.1 M. - 0.16 M. NaCl on Ecteola-SF resin and that the nucleic acid of osmotically shocked  $T_2$  particles was eluted at the higher concentration of 0.3 M. - 0.5 M. NaCl. Bendich et al (1955) showed that calf thymus DNA in highly polymerized form, behaved similarly to RNA with respect to elution and could be eluted from Ecteola-SF cellulose in the range from 0.5 M. - 1.0 M. sodium chloride. So the peak evidenced at a molarity of 0.7 M. sodium chloride on Figure #8 was either cellular DNA, RNA, or DNA from disrupted virus particles (which in part could be responsible for reduced titres on the recovered fractions).







Filtration on Sephadex G200 gel may be a useful method for the initial concentration of Toronto A26/61 virus. As seen in Figure #9, 69% of the original viral infectivity was washed through with the first fraction with subsequent fractions also containing infectivity. Further experiments using this method should be examined, possibly taking electron micrographs of samples of differentially centrifuged preparations before and after the use of the column. Fractions collected from the column would have to be dialyzed to reduce their volume before examination under the electron microscope.

The Tris-genetron extraction procedure, using the method of Green and Pina (1963a), did not result in the concentration of the virus. Absorbance curves of the treated virus were more characteristic of nucleic acid and probably was the nucleic acid released from the cells and from the virus.

The absorbance curve displayed by concentrated Toronto A26/61 virus and shown in Figure #14 could be consistently obtained and was characterized by the lack of a significant increase in absorption at 260 m $\mu$ . in the cases with purified preparations of other animal viruses (Hoyer et al, 1958), a peak at 260 m $\mu$ . was observed.

Green and Pina (1963a) state that the DNA content of adenovirus types 2 and 4 represents only 13% of the total



weight of the virus, which could be a factor in the peculiar absorbance curve displayed by the virus of canine laryngotracheitis.

Gessler et al (1956), in their procedure, used a Virtis homogenizer utilizing a speed of 23,000 rpm. for ten minutes to separate virus and cellular material. Green and Pina (1963a) used a Lourdes multimixer. They homogenized their preparations for one minute at 12,000 rpm. The Toronto A26/61 virus preparation was homogenized for five minutes in a Waring blender at 10,000 rpm. It may be that the virus was incompletely separated from the host cell and the virus that was separated may have been in large clumps. The efficiency of the procedure depends on a fine dispersion of material (Gessler et al 1949). Possibly, therefore, the Toronto A26/61 virus was eliminated with extraneous protein and the nucleic acid from ruptured and dispersed cells was present in the extract -- producing the characteristic absorbance curve when the extract was examined spectrophotometrically. The procedure should be attempted again with closer adherence to the method as described in the original paper.

The buoyant densities of Toronto A26/61 virus and ICH virus are very similar (1.334, 1.330, and 1.333) lending further support to the existence of a close relationship between the two viruses. The low absorbance peaks displayed





by the suspension of ICH virus is due to the paucity of virus in the suspension. However, the infectivity and hemagglutinin were correlated with a buoyant-density of 1.333. The buoyant-density of human adenovirus type 2 (Green and Pina, 1963a) was calculated to be 1.33 approximately. The buoyant-densities of several other viruses are summarized in Table #9.

The relationship between buoyant-density of DNA preparations and the mole fraction % (G + C) would appear to hold true for the Toronto A26/61 virus and the DKL-cell DNA which were examined. Extraction of DNA from Toronto A26/61 virus using (separately) phenol and sodium dodecyl sulfate was attempted several times. When these extracts were examined using buoyant-density-gradient centrifugation the calculated densities were excessive. The buoyant-densities calculated were in a region which would indicate a mole fraction % (G + C) of approximately 80%, which is not possible unless partial denaturation of the DNA had occurred. Sueoka et al (1959) showed that thermal denaturation of DNA from S.marcsens, E.Coli, and calf thymus displayed an increase in buoyant-density measurements.

There was a 3% difference between the mole fraction % (G + C) when the buoyant-density derived mole fraction % (G + C) ( $1.718 = \% / G + C / = 59\%$ ) was compared to chemically derived mole fraction % (G + C) (which was 56%). This





difference appears to be within the range of discrepancies displayed by other DNA extracts when buoyant-density calculations are compared with chemically derived data (see Table #9).

The chemical procedures involved the elucidation of purine and pyrimidine base ratios within the DNA of the Toronto A26/61 virus and also the DKL cell. Five separate analyses were conducted using DKL-cell DNA; but because of the difficulty of obtaining enough Toronto A26/61 virus DNA to do an analysis, only one analysis of Toronto A26/61 virus DNA was completed. Also, poor results were encountered when the virus was submitted to phenol and sodium dodecyl sulfate extraction procedures. Hence, hydrolysis of the intact virus particles was attempted with the subsequent chromatography of this hydrolysate. The base ratios calculated from these procedures are comparable to base ratios of other adenoviruses (see Table #9) and host cell systems (see Table #9) with the exception of the tumorigenic adenoviruses which display a lower guanine plus cytosine ratio (see Table #9) (Green and Pina, 1963b).

Closer examination of the viral DNA should be undertaken before accepting the base ratios revealed in this initial study.



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APPENDIX #1

## Hanks' Balanced Salt Solution -- Hanks and Wallace (1949)

NaCl	8.00 gms./litre
KCl	0.40 " / "
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.20 " / "
KH <sub>2</sub> PO <sub>4</sub>	0.06 " / "
Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O	0.09 " / "
CaCl <sub>2</sub>	0.14 " / "
Glucose	1.00 " / "
NaHCO <sub>3</sub>	0.35 " / "
Phenol Red	0.02 " / "

Designed to equilibrate in a closed bottle at pH 7.2-7.4





APPENDIX #2Earle's Balanced Salt Solution -- Earle et al (1943)

NaCl	6.80 gms./litre
KCl	0.40 " / "
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.20 " / "
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	0.14 " / "
Glucose	1.00 " / "
CaCl <sub>2</sub>	0.20 " / "
NaHCO <sub>3</sub>	0.22 " / "
Phenol Red	0.01 " / "

Designed to equilibrate at pH 7.2-7.6 with 5% CO<sub>2</sub> -  
95% air mixture.



APPENDIX #3

## Eagle's Vitamin Supplement -- Eagle (1955)

Thiamine	1.0 mg./litre
Riboflavin	0.1 " / "
Choline	1.0 " / "
Pyridoxal	1.0 " / "
Folic Acid	1.0 " / "
Inositol	2.0 " / "
Nicotinamide	1.0 " / "
Pantothenate	1.0 " / "

The above supplement is produced and marketed in 100 x concentrations.

The amounts given above are the weights added to a litre of single strength growth medium upon dilution of the supplement to one-fold concentration.



APPENDIX #4

Eagle's Minimal Essential Amino Acids Supplement -- Eagle,  
(1955)

L - Arginine	105 mg./litre
L - Histidine	31 " / "
L - Isoleucine	52 " / "
L - Leucine	52 " / "
L - Lysine	58 " / "
L - Methionine	15 " / "
L - Phenylalanine	32 " / "
L - Threonine	48 " / "
L - Tryptophan	10 " / "
L - Valine	46 " / "
L - Tyrosine	36 " / "
L - Cystine	24 " / "
*L - Glutamine	292 " / "

\*Because of instability in solution, L-Glutamine is omitted and added at time of use (6 ml. of 5% L-Glutamine per litre of single strength medium.)

This supplement is produced and marketed in a 50 x concentration. The weights that appear above are the weights added to one litre of single strength growth medium upon dilution of the supplement to one-fold concentration.





APPENDIX #5Medium 199 -- Morgan et al (1950)

L - Arginine	70 mg.
L - Histidine	20 "
L - Lysine monohydrochloride	70 "
DL - Tryptophane	20 "
DL - Phenylalanine	50 "
DL - Methionine	30 "
DL - Serine	50 "
DL - Threonine	60 "
DL - Leucine	120 "
DL - Isoleucine	40 "
DL - Valine	50 "
DL - Glutamic acid monohydrate	150 "
DL - Aspartic acid	60 "
DL - $\alpha$ -Alanine	50 "
L - Proline	40 "
L - Hydroxyproline	10 "
Glycine (Aminoacetic acid)	50 "
L - Glutamine	100 "
Sodium acetate trihydrate	50 "
L - Cystine	20 "
L - Tyrosine	40 "
L - Cysteine hydrochloride	0.1 "
Adenine	10 "



APPENDIX #5, continued

Guanine	0.3 mg.
Xanthine	0.3 "
Hypoxanthine	0.3 "
Uracil	0.3 "
Thymine (5-methyl uracil)	0.3 "
Disodium a-tocopherol phosphate	0.01"
Thiamine	0.01"
Pyridoxine hydrochloride	0.025 mg.
Riboflavin	0.01 "
Pyridoxal hydrochloride	0.025 "
Niacin	0.025 "
Calcium pantothenate	0.01 "
i-Inositol	0.05 "
Ascorbic acid	0.05 "
Folic acid	0.01 "
p-Aminobenzoic acid	0.05 "
Ferric Nitrate	0.1 "
Biotin	0.01 "
Menadione	0.01 "
Glutathione	0.05 "
Vitamin A	0.1 "
Calciferol	0.1 "
Adenosine triphosphate (disodium salt)	1.0 "



APPENDIX #5, continued

Tween 80	5.0	mg.
Cholesterol	0.2	"
Niacinamide	0.025	"
Adenylic acid	0.2	"
Desoxyribose	0.5	"
D - Ribose	0.5	"
Choline chloride	0.5	"

A chemically defined medium containing the ingredients recommended by Morgan, Morton, and Parker and including their later modifications. This medium is widely used, particularly in the maintenance of tissue for virus production in vaccine manufacture.





APPENDIX #6Preparation of Primary Cell CulturesDog Kidney Cells

Dogs varying in age from two to eight weeks were obtained from the City Dog Pound. They were put to death with chloroform. A midline ventral incision was made into the coelomic cavity skin and subcutaneous tissue were reflected and the kidneys were removed aseptically and were placed into a beaker of Hanks' balanced salt solution containing 100 i.u./ml. of penicillin and 100 ug./ml. streptomycin at pH 7.0. The kidneys were placed in sterile petri dishes and the fibrous capsules were removed. The kidneys were then cut in half laterally and the fibrous tissue forming the calyces of the kidney was removed. The pieces of kidney were then washed in Hanks' balanced salt solution (containing 100 i.u./ml. penicillin and 100 ug./ml. streptomycin) several times. These pieces of kidney tissue were then placed in a sterile 50 ml. beaker, and cut into small (1 mm.) cubes with sterile scissors.

The minced tissue was then added to a 500 ml. trypsinizing flask. 50 ml. of a 0.5% solution of trypsin in phosphate buffered saline at pH 8.0, temperature 37°C, was added to the flask together with a sterile plastic-



APPENDIX #6, continued

covered bar magnet. The flask was placed over a magnetic stirring apparatus and allowed to react until a cloudy suspension of epithelial cells appeared dispersed throughout the trypsin solution. The suspension of cells was decanted into an ice-cooled 250 ml. centrifuge bottle through a sterile gauze filter. The larger cubes of tissue remained in the trypsinizing flask. The centrifuge tube was returned to the ice bath, the temperature of which tends to deactivate the trypsin. A further 50 ml. of trypsin solution was added to the trypsinizing flask and the suspension was allowed to react again. This procedure was repeated until most of the epithelial cells were detached from the small pieces of fibrous capsule which remained behind in the trypsinizing flask.

The cell suspension was centrifuged into a pellet in the International type SB centrifuge using the #259 head at 2,000 rpm. for 15 minutes at 25°C. The trypsin was discarded and the cell pellet was resuspended in fresh nutritive medium (HLA 50 i.u./ml. penicillin and 50 ug./ml. streptomycin and 10% calf serum). The suspension was diluted with nutritive medium until a cell count of 250,000 cells per ml. was obtained and then seeded into Roux tissue culture bottles which were incubated at 37°C until complete monolayers had formed.



APPENDIX #7

## Alsever's Solution -- Kalter (1963)

Dextrose	2.05 gm.
Sodium citrate	0.80 "
NaCl	0.42 "
Citric acid	0.055 "
H <sub>2</sub> O distilled	to make 100 ml.

Sterilize in autoclave, 120°C, 15 psi. 15 minutes. Store at 4°C. Solution remains stable for long periods of time.





APPENDIX #8

## Stain Materials for Tissue Culture -- Humanson (1962)

Zenker's Fixative

Potassium dichromate	2.5 gms.
Mercuric chloride	4.0 - 5.0 gms.
Sodium sulfate	1.0 gms.
Distilled water	100.0 ml.
Glacial acetic acid	5.0 ml.

Lugol's Solution

Iodine crystals	1.0 gms.
Potassium iodide	2.0 "
Distilled water	100.0 ml.

Harris' Hematoxylin

Dissolve 1.0 gm. hematoxylin in 10 ml. ethyl alcohol. Dissolve 20.0 gms. potassium or ammonium alum ( $\text{Al}_2 (\text{SO}_4)_3 \text{K}_2 \text{SO}_4 \cdot 24 \text{H}_2\text{O}$  or  $\text{Al}_2 (\text{SO}_4)_3 (\text{NH}_4)_2 \text{SO}_4 \cdot 24 \text{H}_2\text{O}$ ) in 200 ml. water and boil. Add hematoxylin and boil one-half minute. Add 0.5 gm. mercuric oxide. Cool rapidly. Add a few drops of glacial acetic acid to keep away metallic luster and brighten nuclear structure.

Scott's Solution

Sodium bicarbonate	2.0 gms.
Magnesium sulfate	20.0 "
Distilled water	100.0 ml.
Add pinch of thymol to retard molds	



APPENDIX #8, continuedEosin Counterstain

Eosin Y C I 45380	1.0 gm.
70% ethyl alcohol	1,000.0 ml.
Glacial acetic acid	5.0 ml.

Dilute with equal volume of 70% alcohol for use and add two to three drops of acetic acid.















**B29845**